ISOLATION AND CHARACTERIZATION OF CALMODULIN-LIKE DOMAIN PROTEIN KINASE FROM SOYBEAN

By

JUNG-YOUN LEE

A DISSERTATION PRESENTED TO THE GRADUATE SCHOOL
OF THE UNIVERSITY OF FLORIDA IN PARTIAL FULFILLMENT
OF THE REQUIREMENTS FOR THE DEGREE OF
DOCTOR OF PHILOSOPHY

UNIVERSITY OF FLORIDA

1997

To my parents and Jaewoong

ACKNOWLEDGMENTS

I would like to thank my adviser, Dr. Alice Harmon, for her support, encouragement, and insight into science. Special thanks go to our former lab member, Mrs. Candace MacCafferey, for her technical assistance. I thank my committee members, Drs. Robert Ferl, Don McCarty, Bill Gurley, and David Jones, for their advise and generous sharing of their resources. I owe tremendously to my husband and colleague, Byung-Chun, for his bottomless support and to our jolly son, Jaewoong, who always was willing to let mom go to school. I also cannot thank enough my family in Korea, especially my mother, who was not reluctant to fly over 30,000 miles.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

Abstract of Dissertation Presented to the Graduate School
of the University of Florida in Partial Fulfillment of the
Requirements for the Degree of Doctor of Philosophy

ISOLATION AND CHARACTERIZATION OF CALMODULIN-LIKE DOMAIN PROTEIN KINASE ISOFORMS FROM SOYBEAN

By

Jung-Youn Lee

May, 1997

Chairman: Dr. Alice C. Harmon
Major Department: Plant Molecular and Cellular Biology

Calmodulin-like domain protein kinase (CDPK) is a calcium, but not calmodulin, dependent protein kinase that was first characterized from soybean. CDPKs are encoded by gene families in a wide variety of plant species and are also found in protists. This paper reports the isolation of cDNAs encoding two additional CDPK isoforms from soybean and studies of biochemical characteristics of the recombinant proteins including CDPKα. The new CDPKs with predicted molecular masses of 55 kDa and 60 kDa are named CDPKβ and CDPKγ, respectively, and each showed 76% and 58% amino acid sequence identity to CDPKα. RNA blot hybridization with specific probes for each isoform revealed different expression patterns for each transcript.

For determination of characteristics, the three isoforms were expressed in *Escherichia coli* as glutathione S-transferase fusion proteins and were highly purified. The CDPKs differed in biochemical properties such as stability during storage, requirement of DTT for enzyme activity, and fold-stimulation by calcium. All three isozymes phosphorylated histone IIIS, syntide-2, autocamtide-2, and myosin light chain kinase substrate peptides, but the kinetic parameters of Km_{app} and $Vmax_{app}$, and substrate preferences were different. The pH optimum for all three enzymes was pH 7 to 8. The three soybean CDPKs were inhibited by the general protein kinase inhibitors, K252a and staurosporine at similar or higher IC_{50}s than these for other protein kinases. The protein kinase C specific inhibitor, calphostin C, inhibited the CDPKs with IC_{50}s higher (30- to 200-fold) than that for protein kinase C.

The Ca^{2+}-binding properties of CDPK isoenzymes were studied by flow dialysis in buffer containing 50 mM HEPES, pH 7.5, and 100 mM KCl. The Kds for Ca^{2+} of CDPKα, β, and γ were ~45 μM, 1.5 μM, and 1 μM, respectively. The concentration of Ca^{2+} required for half maximal activity ($K_{0.5}$) of each CDPK ranged from 0.1 μM to 1 μM. The Kd for Ca^{2+} of CDPKα decreased to ~1 μM in the presence of syntide-2 from 45 μM. *In vitro* substrates of CDPK, histone IIIS and Serine acetyl transferase (SAT) affected the Ca^{2+} sensitivity of CDPK activity to different degrees: the $K_{0.5}$s were ~0.1 μM and ~4 μM in the presence of SAT and histone IIIS, respectively. These data demonstrated that each CDPK isoform had unique Ca^{2+}-binding property and kinetics.

These results show that members of the CDPK family differ in biochemical properties and support the hypothesis that each isoform may have a distinct role in Ca^{2+} signal transduction.

CHAPTER 1
LITERATURE REVIEW

Introduction

Ca^{2+} is a versatile intracellular signaling molecule that regulates a wide array of physiological processes. The concentration of cytosolic Ca^{2+} changes in response to a variety of stimuli and results in different cellular responses. A particular physiological response is generated not only by the fluctuation in the level of intracellular Ca^{2+} but also by the route of Ca^{2+} entry and its intracellular localization. The role of Ca^{2+} as a second messenger in signal transduction pathways in animals and plants has been extensively discussed in numerous reviews (Berridge and Dupont, 1994; Bootman and Berridge, 1995; Bush, 1993, 1995; Clapham, 1995; Gilroy et al., 1993; Hepler and Wayne, 1985; Knight et al., 1995; Trewavas and Knight, 1994; Trewavas et al., 1996).

The cytosolic Ca^{2+} concentration is normally maintained at 10-100 nM, and this is over 10,000-fold lower than extracellular Ca^{2+} concentration (1-2 mM). Cells keep cytosolic Ca^{2+} concentration low through numerous binding proteins and specialized pumping proteins because Ca^{2+} is a cytotoxin. At high concentration, Ca^{2+} will form precipitates inside the cell from the reaction with inorganic phosphate. The cytosolic concentration of a closely related divalent cation Mg^{2+} is higher (milimolar) than Ca^{2+} but many cellular processes utilize Ca^{2+} as a signaling molecule. Mg^{2+} binds water more tightly than Ca^{2+}. Since the basal Ca^{2+} concentration is low level and it

has a lower affinity for water than Mg^{2+}, it can act as an intracellular messenger.

The increased Ca^{2+} concentration promotes formation of complexes with target proteins. Ca^{2+}-binding proteins often coordinate Ca^{2+} through ~6 oxygens provided by glutamic acid, aspartic acid, serine, threonine, and asparagine residues (Clapham, 1995; Hepler and Wayne, 1985; Kretsinger, 1987). Most Ca^{2+}-modulated proteins contain EF-hand motifs. The EF-hand consists of two perpendicularly arranged α-helices connected by a Ca^{2+} coordinating loop. EF-hand proteins can be grouped to Ca^{2+} sensors and Ca^{2+} buffers (Ikura, 1996). Ca^{2+} sensors or triggers change their conformations upon binding Ca^{2+} so that they modulate effector molecules. Ca^{2+} buffers, on the other hand, may simply bind Ca^{2+} to keep cytoplasmic Ca^{2+} level low. Due to the number, affinity, and specificity of Ca^{2+}-binding proteins in cells, the Ca^{2+} signal can be highly localized. It is estimated that the effective range of free Ca^{2+} is 0.1 μm lasting only ~30 μs before it is buffered, and that of buffered Ca^{2+} is ~5 μm lasting ~1 s provided that the on-rate of a typical calcium buffer is 108 $M^{-1}s^{-1}$ and the concentration of Ca^{2+} buffers is ~300 μM (Allbritton, et al., 1992). The slow diffusion of Ca^{2+} results from its binding to slowly mobile or immobile buffers. Since the rapid buffering of Ca^{2+} makes it a localized messenger, the effector molecules that require a high concentration of Ca^{2+} for activation have to be located less than ~0.5 μm from a Ca^{2+} source.

As a second messenger, Ca^{2+} signals exhibit highly organized temporal and spatial arrangement. The spatiotemporal aspect of Ca^{2+} signaling is complex and the underlying mechanism is not yet fully defined (Berridge and Dupont, 1994; Miyazaki, 1995).

Generation of Changes in Cytosolic Ca^{2+} Level

In plants, cytosolic Ca^{2+} levels have been reported to rise upon various extracellular stimuli such as abscisic acid (Allan et al., 1994; Gilroy, 1996; McAinsh et al., 1992; Zocchi and De Nish, 1996), auxin (Felle, 1988; Gehring et al., 1990), gibberellic acid (Bush, 1996; Gilroy, 1996), cold shock (Knight et al., 1991; Knight et al., 1996; Monroy and Dhindsa, 1995), light (Bowler et al., 1994; Fallon et al., 1993; Gehring et al., 1990; Nauhaus et al., 1993; Shacklock and Trewavas, 1992), gravity (Gehring et al., 1990), mechanical stimulation (Knight et al., 1991; Knight et al., 1992), osmotic stress (Cramer and Jones, 1996), anoxia (Sedbrook et al., 1996; Subbaiah et al., 1994), oxidative stress (McAinsh et al., 1996; Price et al., 1994), elicitors (Knight et al., 1991), and pathogen (Levine et al., 1996). Ca^{2+} is also involved in pollen tube growth (Franklin-Tong et al., 1996; Malho and Trewavas, 1996; Pierson et al., 1994), root nodule formation (Ehrhardt et al., 1996), ethylene (Philosoph-Hadas et al., 1996; Raz and Fluhr, 1992), and carbon dioxide (Webb et al., 1996) signal transduction pathways. Changes in cytosolic Ca^{2+} are essential to these processes and the changes are highly variable in amplitude, kinetics, and spatial distribution. Changes in cytosolic Ca^{2+} induced by different stimuli may be transient, sustained, or oscillatory and the time that required for these changes varies from seconds to hours. In plants, rapid and transient changes in cytosolic Ca^{2+} could be induced by abscisic acid (ABA), auxin, cold, red light, elicitor, and mechanical stimuli while slower and more complex changes in cytosolic Ca^{2+} were observed in responses induced by auxin, ABA, red light, gibberellic acid (GA). Changes in cytosolic Ca^{2+} also differ with respect to spatial characteristics (Bush, 1996). A steady state Ca^{2+} gradient across the plasma membrane is present in pollen tubes (Pierson et al., 1994)

and root hairs (Felle et al., 1992). It was suggested that these Ca^{2+} gradients were formed based on the extracellular Ca^{2+} influx into cytoplasm through the activity of Ca^{2+} channels at the apical plasma membrane. In addition, the intracellular Ca^{2+} gradient might be regulated by specific pumps that extrude Ca^{2+} from cytoplasm to lower the Ca^{2+} concentration to basal levels (Pierson et al., 1994).

ABA and Stomatal Closure

The plant hormone ABA was first discovered as a naturally occurring compound that accelerates leaf abscission and bud dormancy in woody plants. ABA is also involved in other processes such as seed dormancy and germination, the regulation of stomatal aperture, and the responses to environmental stress. Plants respond to excessive water loss by controlling the pore size of stomata. ABA stimulates stomatal closing by increasing efflux of K^+ and anions from guard cells (Giraudat, et al., 1994).

The aperture of stomatal pores through which the exchange of CO_2 and water vapor occurs is controlled by the two surrounding guard cells. Guard cell volume changes in response to many signals such as CO_2, humidity, phytohormones, and light (Kearns, E.V. and Assmann, S.M., 1993). ABA is a well known phytohormone to induce stomatal closure during water stress. It has been reported that ABA induces rapid, transient increases (Gilroy, et al., 1991; McAinsh et al., 1992) or repetitive increases (Schroeder and Hagiwara, 1990) of cytosolic Ca^{2+} prior to stomatal closure.

Increases of transient cytosolic Ca^{2+} were observed with both open and closed guard cells of *Commelina communis* in response to ABA treatment (Gilroy, et al., 1991; McAinsh et al., 1992). Fluorescence ratio imaging of ABA-stimulated guard cell using microinjected calcium indicator Indo-I

demonstrated that cytosolic Ca^{2+} increases were not uniformly distributed but localized in the periphery of the cell and the external boundaries of internal Ca^{2+} stores. Gilroy et al. (1990) microinjected fluorescent Ca^{2+} indicator Fluo-3 and caged Ca^{2+} and monitored changes in stomatal aperture of *Commelina communis*. When cytosolic Ca^{2+} was elevated above 600 nM by UV photolysis of caged Ca^{2+}, stomatal closure was observed. When caged inositol-1, 4, 5-trisphosphate ($InsP_3$) was photoactivated or $InsP_3$ was directly microinjected, increases of cytosolic Ca^{2+} from intracellular Ca^{2+} stores were observed followed by subsequent stomatal closure. However, the induction of cytosolic Ca^{2+} increases by ABA treatment were highly variable while all stomata were closed in response to ABA (Gilroy, et al., 1991; McAinsh et al., 1992). These observations led to the question of whether ABA-induced stomatal closure occurs through both Ca^{2+}-dependent and Ca^{2+}-independent pathways. Allan et al. (1994) investigated the effects of growth temperature on ABA-induced cytosolic Ca^{2+} changes and showed that ABA signal transduction in guard cells resulting in stomatal closure could be mediated by both Ca^{2+}-dependent and Ca^{2+}-independent pathways. The guard cells of *Commelina* plants grown at 10-17 °C did not show an increase in cytosolic Ca^{2+} by ABA treatment although all of them were closed. In contrast, when the plants were grown at 25 °C an increase in cytosolic Ca^{2+} was always accompanied by ABA-induced stomatal closures. Elevation of cytosolic Ca^{2+} in response to ABA treatment was also impaired when the plants were pre-exposed to water stress.

ABA also has been implicated in inducing repetitive increases of cytosolic Ca^{2+} in *Vicia faba* guard cells using patch clamping and fluorescent imaging with Fura-2, (Schroeder and Hagiwara, 1990). The increase of Ca^{2+} occurred simultaneously with the openings of nonspecific cation channel in

the plasma membrane. The result indicated that Ca^{2+} enters the cell through these channels. The activation of these nonselective, Ca^{2+}-permeable ion channels occurred within 2 seconds of ABA treatment. When cytosolic Ca^{2+} was elevated to micromolar, inward K^+ channel activity at the plasma membrane was inhibited and caused anion efflux (Schroeder and Hagiwara, 1989; 1990) which resulted in membrane depolarization and activation of outward K^+ channels in the plasma membrane. The net efflux of ions from the stomatal guard cells caused reduction in turgor and closing of stomata. Increases in cytosolic Ca^{2+} in guard cells also showed the activation of outward vacuolar K^+ channels (Ward and Schroeder, 1994). The activation of vacuolar K^+ channels resulted in depolarization of vacuolar membrane which is sufficient to activate slow vacuolar ion channels. These slow vacuolar ion channels revealed a permeability ratio for Ca^{2+} to K^+ of ~3:1 suggesting a possible mechanism for Ca^{2+}-induced Ca^{2+}-release from vacuoles during stomatal closure. Gilroy et al. (1990) showed $InsP_3$ could induce cytosolic Ca^{2+} increase and subsequent stomatal closure, when caged $InsP_3$ was microinjected into the guard cells and was activated by photolysis. Whether $InsP_3$ is the *in vivo* mediator of Ca^{2+} release from vacuoles requires further examination. Luan et al. (1993) proposed that the inactivation of inward K^+ channels on the plasma membrane in guard cells may be mediated by the Ca^{2+}/calmodulin dependent protein phosphatase 2B (PP2B) homolog using a specific inhibitor of PP2B. Also, when the constitutively active (Ca^{2+}-independent) form, i.e., catalytic subunit of PP2B was introduced into the guard cell, inward K^+ channels were inhibited in the absence of Ca^{2+}. Fairley-Grenot and Assmann (1991) reported that heterotrimeric G-protein may be involved in stomatal closure through the regulation of K^+ channels. When nonhydrolyzable GDP analogue, GDPβS was introduced into the cytosol of

Vicia faba guard cells, inward K^+ current through the plasma membrane was activated. On the contrary, the GTP analogue, GTPγS inhibited the inward K^+ current. However, in the presence of Ca^{2+} chelator BAPTA, GTPγS did not inhibit the current implying cross talk between G-protein signaling and Ca^{2+} signaling in the regulation of stomatal aperture.

GA and Secretion in Aleurone Cells

The physiological role of endogenous GA is well documented in germinating cereal seeds. When seeds imbibe water, GA is synthesized by the embryo and translocated to the cells in the aleurone layer which surrounds the starch-storing cells in endosperm. GA stimulates the synthesis of hydrolases from the aleurone. The enzymes are then secreted into the endosperm where starch breakdown occurs (Gilroy et al., 1993). The most prominent hydrolase that is secreted upon stimulation by GA is the Ca^{2+} containing metaloprotein α-amylase. GA is perceived at the plasma membrane and the signal is transduced by Ca^{2+}-dependent and independent pathways (Bush, 1996; Gilroy, 1996).

GA induced a sustained increase in cytosolic Ca^{2+} at the periphery of the barley aleurone cell protoplasts from 100 nM to above 600 nM. The increase started 1 to 4 hours after GA treatment and lasted up to 8 hours, although individual protoplast differed in response kinetics (Gilroy, 1992; 1996). The increase of cytosolic Ca^{2+} and α-amylase secretion induced by GA was dependent on extracellular Ca^{2+}. Gilroy and Jones (1993) observed that calmodulin levels were increased in aleurone layers by GA treatment before hormone-induced α-amylase synthesis and secretion. Application of calmodulin stimulated Ca^{2+} transport into ER of aleurone cells as observed in those cells treated with GA implying calmodulin as a possible mediator in GA

signal transduction pathways. Wheat aleurone cells showed similar levels of cytosolic Ca^{2+} increase, but the response was faster; the Ca^{2+} increase was initiated within a few minutes and fully developed after 0.5 to 1.5 hours (Bush, 1996). The increase in cytosolic Ca^{2+} induced by GA correlated with the GA responses of barley aleurone protoplasts; changes in vacuolar morphology, activation of α-amylase gene transcription, and amylase secretion. However, mimicking the prolonged cytoplasmic Ca^{2+} increase by microinjection or activating caged Ca^{2+} instead, did not mimic GA action in activation of α-amylase gene. Only α-amylase secretion was blocked when caged Ca^{2+} chelator was used in the GA stimulated protoplasts. These results suggested that GA signal transduction is similar to ABA signaling in that there appears to be Ca^{2+}-dependent and Ca^{2+}-independent pathways (Gilroy, 1996). The effect of GA on the increase of cytosolic Ca^{2+} was reversed by ABA treatment and resulted in inhibition of α-amylase secretion (Gilroy, 1992). However, microinjection of Ca^{2+}/calmodulin into the barley aleurone protoplasts blocked ABA inhibition of all of the GA-induced responses, i. g., both α-amylase secretion and gene transcription (Gilroy, 1996). These results suggested involvement of other signaling elements integrated into the ABA-induced inhibition of Ca^{2+}-dependent and Ca^{2+}-independent GA action.

Mechanical Signaling

Mechanical signals exert significant effects on the development and morphology of plants. Externally applied mechanical signals such as touch and wind induce immediate elevation of cytosolic Ca^{2+} in plants. Knight, et al. (1991, 1992) demonstrated the effect of mechanical signals on changes of cytosolic Ca^{2+} by utilizing transgenic tobacco plants expressing the Ca^{2+}-sensitive protein aequorin. Aequorin is a bioluminescent protein found in

the jelly fish *Aequorea victoria*. It consists of an apoprotein with a molecular mass of 22 kDa and a small hydrophobic lumiphore, coelenterazine. Upon binding Ca^{2+} aequorin emits a photon of blue light and the coelenterazine become oxidized and inactivated (Blink et al., 1989). The aequorin-coding sequence was fused to the cauliflower mosaic virus (CMV) 35S promoter and constitutively expressed in tobacco plants (Knight, et al., 1991). Over 97% of aequorin was found in soluble fractions of homogenates of transgenic seedlings. When cotyledons of transgenic tobacco seedlings were touched and monitored in a luminometer, an intracellular Ca^{2+} spike was observed with each touch. A transient increase of cytosolic Ca^{2+} was also induced in transformed seedlings by stimulating them with blasts of air from a syringe. The mesophyll protoplasts and epidermal strips isolated from the transgenic tobacco plants responded by increasing cytosolic Ca^{2+} up to 10 μM when stimulated by injecting an isotonic medium (Haley et al., 1995).

Stimulation by wind or touch brings about the expression of *TCH* genes (Braam and Davis, 1990). These *TCH* genes encode calmodulin and calmodulin homologous proteins. *Arabidopsis* plants stimulated by touch showed inhibited elongation of petiole and bolt compared to unstimulated plants. *TCH* gene expression was also induced within 10 to 30 minutes by treating cultured root cells of *Arabidopsis* with 100 mM $CaCl_2$ (Braam, 1992). Although changes in intracellular Ca^{2+} may transduce the mechanical signals, the mechanism whereby touch and wind stimuli modify plant growth and development is not understood.

Red Light Response

Red light induces transient increase of cytosolic Ca^{2+} and leads to the swelling of etiolated wheat leaf protoplast (Shacklock et al., 1992). This

response can be mimicked by photorelease of caged Ca^{2+} or caged $InsP_3$. Transient elevation of cytosolic Ca^{2+} that lasted less than 1 minute was sufficient to induce the increase of protoplast volume by 20% within 10 minutes. When red-light irradiation was followed by a subsequent far-red light treatment, the effect of red-light on the transient Ca^{2+} change was reversed. Fallon et al. (1993) observed that red-light exposure increases phosphorylation of proteins in etiolated wheat leaf protoplasts and this response is also induced by photolysis of caged Ca^{2+}. Protein phosphorylation was enhanced by increasing extracellular Ca^{2+}, but decreased by increasing a Ca^{2+} chelator, EGTA, (ethylene glycol bis(β-aminoethyl ether)-*N,N,N",N"*-tetraactic acid).

Using phytochrome deficient tomato cells, Neuhaus et al. (1993) proposed that phytochrome signal transduction is mediated by Ca^{2+}/calmodulin-dependent and -independent pathways. Phytochrome is a soluble protein with a chromophore which absorbs red and far-red light. This photoreceptor exists in two forms; Pfr (far-red absorbing form) and Pr (red light absorbing form), which are interconvertible by the appropriate irradiation (Quail, 1991). A phytochrome-deficient tomato mutant was able to develop the maturation of chloroplasts and biosynthesis of anthocyanin pigment when microinjected with activated G-protein (Neuhaus et al., 1993). However, Ca^{2+} and calmodulin could induce only the development of chloroplasts while cyclic GMP could trigger anthocyanin synthesis in the phytochrome-deficient tomato mutant (Bowler, et al., 1994). These mediators of the phytochrome signal transduction pathway are shown to modulate the expression of light responsive genes.

Ca^{2+} Oscillations

In animal systems, Ca^{2+} signaling has been categorized in two groups: signaling in electrically excitable cells, such as nerve and muscle, and in inexcitable cells, such as epithelial or blood cells (Clapham, 1995; Ghosh and Greenberg, 1995; Hardie, 1996; Putney, 1993). Both groups of cells utilize mobilized Ca^{2+} from Ca^{2+}-sequestering compartments inside cells. In excitable cells, Ca^{2+} signaling is mediated by so called, Ca^{2+}-induced Ca^{2+}-release (CICR) mechanism whereas in inexcitable cells, it is mediated by the capacitative Ca^{2+} entry mechanism.

In inexcitable cells, Ca^{2+} signaling is typically a biphasic process: extracellular signals stimulate an intracellular organelle to release stored Ca^{2+} into the cytoplasm, and this Ca^{2+} efflux from intracellular organelles induces more Ca^{2+} influx into the cytoplasm from the extracellular space (Putney, 1993; Clapham, 1995). G-protein coupled receptors or receptor protein kinases lead to activation of phospholipase C on the plasma membrane upon agonist binding. Activated phospholipase C generates $InsP_3$ which in turn, diffuses to specific receptors on the ER. The $InsP_3$ receptor is a ligand activated, Ca^{2+}-selective channel. The $InsP_3$ receptor releases Ca^{2+} upon binding to $InsP_3$ inducing transient cytoplasmic Ca^{2+} increase and depletion of the intracellular Ca^{2+} store. The empty Ca^{2+} store generates a retrograde signal that activates Ca^{2+} influx across the plasma membrane resulting in capacitative Ca^{2+} entry (Putney, 1993). The capacitative Ca^{2+} entry is also called, store-operated Ca^{2+} entry. An electrical current associated with this entry is called the "calcium release-activated calcium current" (I_{CRAC}). I_{CRAC} was shown to increase by a novel calcium influx factor, CIF (Clapham, 1993; Randriamampita and Tsien, 1993; Parekh, et al., 1993). CIF is a small non-

peptide molecule generated by cells whose Ca^{2+} stores have been depleted and move from organelle to cytoplasm. It can even move across the plasma membrane and stimulate adjacent cells. However, the identification of CIF still remains elusive. The mechanism by which the depleted stores signal the Ca^{2+}-influx channels (store-operated channels) is not clear, but the store operated channels in inexcitable cells are not regulated by membrane potential unlike the Ca^{2+} channels in excitable cells.

In excitable cells, the $InsP_3$-triggered mechanism or the CICR mode of the signaling pathway may operate. However, virtually all excitable cells contain voltage-activated Ca^{2+} channels and many excitable cells express the ryanodine receptor. Ryanodine receptors are gated either by electromechanical coupling to the dihydropyridine receptor of plasma membrane in skeletal muscle, or by Ca^{2+}, or by cyclic ADP-ribose (cADPR) in some other cell types. Depolarization of the cytoplasmic membrane activates voltage-dependent Ca^{2+} channels thus enabling the flood of Ca^{2+} across the membrane. However, the voltage-dependent Ca^{2+} channel activity is also time-dependent. Ca^{2+} entering through voltage-dependent channels may directly activate ryanodine receptors to release Ca^{2+} from intracellular stores via CICR (Clapham, 1995). cADPR activates directly the cardiac but not skeletal ryanodine receptor and calmodulin can modulate the action of cADPR on ryanodine receptor activation (Ghosh, and Greenberg, 1995).

In excitable cells, CICR amplifies the magnitude and spatial distribution of the transient Ca^{2+} signal for rapid, all-or-none responses. In inexcitable cells, the capacitative Ca^{2+} entry amplifies the duration of the Ca^{2+} signal, leading to sustained or tonic responses. When cells are activated through the capacitative mechanism, the level of Ca^{2+} often oscillates with a frequency that varies depending on the concentration of agonist. This regenerative

mechanism enables each Ca^{2+} pulse to spread throughout the cytosol as a Ca^{2+} wave. It is thought that such frequency-encoded Ca^{2+} pulses may convey more information than the simple static increase of cytosolic Ca^{2+} (Putney, 1993).

In plants, Ca^{2+} oscillations have been recently observed in the responses stimulated by nodulation factors (Ehrhardt et al., 1996), osmotic changes (Taylor, et al., 1996), and pistil S proteins (Franklin-Tong et al., 1996). Cytosolic Ca^{2+} oscillation induced by extracellular Ca^{2+} in guard cells of *Commelina communis* (McAinsh et al., 1995). Allen et al. (1995) demonstrated the presence of both $InsP_3$ receptors and ryanodine receptors in plants. Vacuolar Ca^{2+} was released in response to ryanodine and cADPR. Patch-clamping data showed that the cADPR receptor was voltage sensitive, and spontaneous inactivation of the receptor was not observed. Moreover, ryanodine receptors and $InsP_3$ receptors were shown to colocalize in the vacuolar membrane. It is suggested that the inactivation of these channels may depend on the ligand metabolism in plants due to the high volume of the vacuoles (~80%) which contains milimolar Ca^{2+}, unlike in animal cells where the termination of the ligand-gated Ca^{2+} signal arises from the depletion of internal stores.

Ca^{2+} signaling is shown to be involved in mediating the self-incompatibility response in pollen of *Papaver rhoeas* (Franklin-Tong.et al., 1995). Self-incompatibility is a mechanism which regulates the acceptance or rejection of pollen on the pistil. It is controlled by genes at the S-locus. Self fertilization is inhibited when pollen carries identical S-allele to that of pistil. Transient elevation of cytosolic Ca^{2+} was observed when challenged with self-incompatibility (S-) protein and was followed by inhibition of pollen growth. The increase of cytosolic Ca^{2+} was spatially localized in the intracellular

region associated with the nucleus and ER. Using photoactivated caged $InsP_3$, Franklin-Tong et al. (1996) demonstrated that $InsP_3$ could induce intracellular Ca^{2+} release starting behind the tip of the pollen tube and expanding toward the tip as a form of Ca^{2+} wave. The cytosolic Ca^{2+} increase induced by $InsP_3$ showed slow transient kinetics increasing for 5 minutes and lasting about 6 minutes. In parallel, Ca^{2+}-dependent protein phosphorylation of a 26 kD pollen protein was induced by the self-incompatibility response implying the involvement of a Ca^{2+}-regulated protein kinase in this signaling (Rudd et al., 1996).

In response to compatible *Rhizobium* nodulation factor, alfalfa root hairs showed an asymmetric Ca^{2+} oscillation in the form of baseline spikes (Ehrhardt, et al., 1996). These Ca^{2+} spikes initiated 9 minutes after the root hairs were treated with Nod factors and continued up to 3 hours. The initiation of the Ca^{2+} elevation and spiking pattern were observed in the nucleus region and propagated into the cytoplasm toward the root hair tip. The Ca^{2+} spike induced by nodulation factor was specific to compatible host plants, e. g., a nonlegume plant or alfalfa mutant lacking the nodulation response failed to show changes in cytosolic Ca^{2+}. In animals, the frequency but not the amplitude of baseline Ca^{2+} spikes is determined by agonist concentration. On the other hand, the agonist concentration regulates the amplitude but not the frequency of sinusoidal Ca^{2+} oscillation. These phenomena, which are observed in many cell types and in response to different stimuli are thought to be regulated by distinct Ca^{2+}-dependent processes (Thomas et al., 1996).

Regulation of Cytosolic Ca^{2+}

Like other organisms plant cells maintain the cytosolic free Ca^{2+} at very low concentration under resting conditions (Bush et al., 1996). The transport of Ca^{2+} across cellular membranes are controlled by Ca^{2+}-influx and -efflux transporters. Increase in cytosolic Ca^{2+} induced by a stimulus is accomplished either by an influx of extracellular Ca^{2+} through Ca^{2+} channels in the plasma membrane or by the release of Ca^{2+} from intracellular Ca^{2+} stores.

The role of Ca^{2+} as a second messenger is the transmission of extracellular signals perceived by localized receptors to other parts of the cell where effector molecules of the response reside. The increased Ca^{2+} in the cytoplasm binds Ca^{2+} modulated proteins and activates them which, in turn results in the regulation of target protein activities to generate specific responses. The signal transduction cascade mediated by cytosolic Ca^{2+} starts from the perception by the cell. This signal perception leads to the regulation of Ca^{2+} transporters and results in changes of an intracellular Ca^{2+} level. It is well known in animal systems that the generation of intracellular Ca^{2+} signal is often regulated by a second messenger $InsP_3$. In response to many stimuli, both $InsP_3$ and diacylglycerol are formed, and the $InsP_3$ is released into cytoplasm to mobilize Ca^{2+} from internal stores (Berridge, 1993). The formation of $InsP_3$ is mediated by G-protein-linked receptors or by receptor tyrosine kinases. Signaling cross-talk between the second-messenger generating systems generate the complexity of Ca^{2+} signals.

G-protein Mediated Cytosolic Ca^{2+} Regulation

In animal cells, G-proteins couple the receptors and the signaling systems which generate various second messenger in order to induce specific

cellular responses upon various stimuli and often induce changes in cytosolic Ca^{2+}. Many plasma membrane receptors when activated, generate more than one second messenger involving cyclic AMP and/or $InsP_3$ by activating multiple G-proteins. Heterotrimeric G-proteins are part of the larger GTPase superfamily that also includes small GTP-binding proteins. Heterotrimeric G-proteins consist of an α subunit which binds and hydrolyzes GTP, and β and γ subunits. The β and γ subunits forms an inseparable, functional monomer. When GDP is bound, α subunit associates with the βγ subunits forming an inactive heterotrimer that binds to the receptor. Upon stimulation, the receptor becomes activated and changes its conformation. The GDP-bound α subunit then responds with a conformational change that favors GTP binding. Once bound to GTP, the α subunit dissociates from βγ subunits and the receptor, and hydrolyzes GTP. The free α and βγ subunits each activate target effectors. GTP hydrolysis is a time control that determines how long both activated α and βγ subunits last (Neer, 1995; Rens-Domiano and Hamm, 1995).

Heterotrimeric G-proteins are an important component of signal transduction pathways since they transfer information perceived at the cell surface to the downstream effector molecules such as adenylate cyclases and phospholipase C (PLC). PLC hydrolyzes phosphatidylinositol phosphates to generate $InsP_3$ and diacylglycerol. $InsP_3$ then induces release of intracellular Ca^{2+} and Ca^{2+} further modulates the activities of PLC (Berridge, 1993). Also the fact that both α subunit and βγ subunits can modulate PLC greatly increase the potential complexity of signal transduction. These multiple signaling cross-talks make defining steps in signal transduction difficult. However, these mechanisms provide complex and fine modulation of cytosolic Ca^{2+} for divers cellular responses (Fyfe and Roberts, 1995).

In plants, the possible involvement of heterotrimeric G-proteins has been implicated in diverse physiological processes. Among these is the proposed G-protein regulation in guard cell physiology (Armstrong and Blatt, 1995; Fairley-Grenot and Assmann, 1991; Kelley, et al., 1995; Lee et al., 1993). These authors used GTP analogues to monitor their effect on K^+ channel activity or stomatal opening. However, the results are not consistent and quite complex rendering them difficult to interpret. For better understanding of the role of G-protein in plants, isolation of the genes especially expressed in guard cells and biochemical characterization seem to be prerequisite (Assmann, 1996).

Ca^{2+} Channels

In animals, it is well documented that Ca^{2+} signals are generated from two sources; Ca^{2+} influx across the plasma membrane and Ca^{2+} release from intracellular stores (Berridge and Dupont, 1994; Bootman and Berridge, 1995; Clapham, 1995; Miyazaki, 1995). Ca^{2+} entry across the plasma membrane is through the opening of plasma membrane Ca^{2+} channels that are voltage-gated or receptor-operated. Ca^{2+} efflux from intracellular stores is controlled by two types of Ca^{2+} release channels; $InsP_3$ receptor channels and ryanodine receptor channels. The coordination of these channels provide spatial and temporal regulation of the elevation in cytosolic Ca^{2+} levels and can give rise to highly localized Ca^{2+} signals; periodic opening and closing of the calcium channels brings about repeated Ca^{2+} spikes or oscillations and Ca^{2+} waves.

The $InsP_3$ receptor forms a homotetramer composed of subunits of approximately 310 kDa, and one $InsP_3$ binds each subunit. The primary sequence of the $InsP_3$ receptor shares no homology with proteins forming the Ca^{2+} channels on the plasma membrane but shares partial homology with the

ryanodine receptors in muscles. Ligand binding and regulatory domains reside in the cytoplasmic region. This region is enriched in basic residues and binds heparin. The transmembrane domain has six or eight membrane-spanning regions. Various isoforms are result from alternative splicing and separate genes. ATP binds the receptor and enhances the channel activity. The activity of the receptor is also modulated by protein kinase A, protein kinase C, and calmodulin-dependent protein kinase II. Ca^{2+} itself has been shown to bind the receptor. $InsP_3$-induced Ca^{2+} flux is described by a bell-shaped curve depending on the concentration of Ca^{2+}; i. e., at low and high Ca^{2+} levels, the $InsP_3$ receptor is relatively insensitive to $InsP_3$ suggesting that $InsP_3$-induced Ca^{2+} release is regulated by Ca^{2+} (Mikoshiba, 1993).

The ryanodine receptor-Ca^{2+} channel complex consists of a homotetramer of 550 kDa, and several isoforms have been identified. Ca^{2+} is the primary activating ligand of ryanodine receptors in skeletal muscle (RY1) and cardiac muscle (RY2) among other modulators such as ryanodine, caffeine, cyclic ADP-ribose, ATP, and calmodulin. However, the mechanisms governing the activation of ryanodine receptors are isoform and cell type dependent. RY1 is gated by electromechanical coupling to the plasma membrane dihydrophyridine receptor, and RY2 is gated by Ca^{2+}-induced Ca^{2+} release (Sitsapesan, et al., 1995). The activities of ryanodine receptors are also regulated by phosphorylation by protein kinase A (Valdivia, et al., 1995) and calmodulin-dependent protein kinase II (Takasawa, et al., 1995).

Observations suggesting the presence in plants of the Ca^{2+} oscillators similar to those found in animals are accumulating (Bush, 1995). Three types of Ca^{2+} channels have been identified by their electrophysical characteristics in plants; voltage-gated, ligand-gated, and mechanically operated. Voltage-gated Ca^{2+} channels are present both in the plasma membrane (Pineros and

Tester, 1995; Thuleau et al., 1994) and in the vacuolar membrane (Allen and Sanders, 1994, 1995; Johannes and Sanders 1995; Ward and Schroeder, 1994). $InsP_3$-gated and cyclic ADP-ribose-gated Ca^{2+} channels are present in the vacuolar membrane (Allen et al., 1995). Biswas et al. (1995) recently purified a protein from microsomal fractions of mung bean using heparin affinity chromatography which was shown to be reconstituted to yield $InsP_3$-gated Ca^{2+}-release activity. It forms a homotetramer (110 kDa monomer) and is markedly different from its mammalian counterpart (~250 kDa). The presence of $InsP_3$-elicited Ca^{2+} release channels at plant vacuoles may imply a role for $InsP_3$ as a second messenger in plants. At present there is no molecular evidence for the existence of a ryanodine-like Ca^{2+} release channel in plants. In storage roots of red beet, cyclic ADP-ribose has been shown to elicit Ca^{2+} release from vacuole-enriched microsomes (Allen et al., 1995). Muir and Sanders (1996) showed that the Ca^{2+} release induced by cyclic ADP-ribose from red beet microsomes is comparably sensitive to modulation by ryanodine receptor agonists and antagonists as shown in animal cells. In addition to the ligand-gated Ca^{2+} release channels, another class of Ca^{2+} channel resides at the vacuolar membrane. These channels, which are apparently not identified in animals, are activated by hyperpolarization of the vacuolar membrane (Allen and Sanders, 1994; Johannes and Sanders, 1995). However, the physiological role of these channels is not known.

Depolarization-activated (voltage-gated) channels in vacuoles are known as the slowly activating vacuolar channels (SV channels), which activates in response to the depolarizing potentials over one hundred milliseconds (Allen and Sanders, 1995; Ward and Schroeder, 1994). SV channels are gated open by a physiological range of cytosolic Ca^{2+} (0.1-1 μM) as well as by depolarization (Sanders et al., 1995). These channels seem to be

regulated by calmodulin (Bethke and Jones, 1994) and calcineurin (Allen and Sanders, 1995). The opening of SV channels in barley aleurone cells was sensitive to cytosolic Ca^{2+} in a range of relatively high concentration (600 nM-100 μM) and the activity was inhibited by calmodulin inhibitors (W7 and trifluoperazine). Adding calmodulin reversed partially the effects of the inhibitors. Calmodulin also sensitizes the channel to cytosolic Ca^{2+} in a range of 2.5 to 10 μM (Bethke and Jones, 1994). GA treatment of aleurone protoplasts increased the specific current compared to protoplasts treated with $CaCl_2$ or ABA. The activity of SV channels from broad bean guard cells was not modulated by calmodulin (Allen and Sanders, 1995). But it was potentially modulated by calcineurin, i. e., at high concentration calcineurin showed strong inhibition of the channel activity. It was suggested that modulation of SV channel activity by calcineurin may play a role as feedback inhibition. The SV channel could mediate CICR because the channel can be activated by increases in cytosolic Ca^{2+} (Ward and Schroeder, 1994) and vacuoles contain high concentrations of Ca^{2+} which is virtually inexhaustible. Therefore, tight regulation of CICR will be required in plants. The model proposed by Allen and Sanders (1995) is that the initial rise of cytosolic Ca^{2+} opens SV channels, which then releases Ca^{2+} that activates Ca^{2+}/calmodulin dependent phosphatase, calcineurin, leading to the feed back inhibition of the channels. The presence of different types of Ca^{2+} release channels in plant cells may contribute to the generation of complex temporal and spatial Ca^{2+} signals in response to various stimuli similar to those demonstrated in animal cells.

Ca^{2+} Modulated Proteins

Elevation of cytosolic Ca^{2+} is detected by Ca^{2+} sensors that play roles in altering activities of enzymes, pumps, and other targets. Ca^{2+} binding proteins are known as EF-hand proteins, although not all Ca^{2+} binding proteins contain an EF-hand motif (Kretsinger, 1996). To date there are 41 known subfamilies of EF-hand proteins (Kretsinger, 1996). The structural motif of the EF-hand was first discovered from the crystal structure of parvalbumin (Kretsinger, 1987). Parvalbumin contains three calcium-binding domains designated AB, CD, and EF from N-terminus to C-terminus. The name EF-hand (or calmodulin fold) is originated from the domain EF which forms a α-helix-loop-α-helix structure. The Ca^{2+} is coordinated by the loop which contains 12 amino acid residues and provides oxygen atoms from side chains of five amino acids to coordinate the Ca^{2+}. Five oxygen atoms are provided by residues 1, 3, 5, 7, and 9, and two are provided by a glutamate at position 12 (Babu et al., 1988). Residue 7 coordinates Ca^{2+} by a peptide carbonyl group. The EF-hand usually works as a pair, but different EF-hand subfamilies contain different numbers of EF-hands ranging from two to eight.

Ca^{2+} binding proteins are classified as trigger or buffer proteins based on their affinity for Ca^{2+} (Ikura, 1996). Buffer proteins have higher affinity than sensor proteins; thus, they are thought to respond to even a very small fluctuation of cytosolic Ca^{2+} and sequester Ca^{2+} to maintain a low cytosolic Ca^{2+} concentration. However, a possible trigger function for buffer proteins remains to be elucidated. The best known Ca^{2+} trigger protein is calmodulin, and its physiological roles are almost as diverse as those of Ca^{2+} (Cheung, 1980). Calmodulin (17 kDa) is involved in cyclic nucleotide metabolism, phosphorylation cascade, cell division, Ca^{2+} transport system, and

dephosphorylation in animals (James et al., 1995). The main protein targets of calmodulin are phosphodiesterase, adenylate cyclase, calmodulin-dependent kinase I and II, elongation factor kinase, myosin light chain kinase, phosphorylase kinase, plasma membrane Ca^{2+} pump, and calcineurin. Other Ca^{2+} sensors include troponin C, calcineurin B, myosin light chains, recoverin, S100 proteins, and visinin. The molecular size of these proteins range from 18 kDa to 22 kDa and contain 2 or 4 EF-hands (Ikura, 1996). Ca^{2+} buffer proteins include parvalbumin, calbindin D, and calretinin. Structural studies showed that Ca^{2+}-binding proteins that undergo large conformational changes upon binding Ca^{2+} are all known to have a trigger function in the activation of target proteins. In contrast, Ca^{2+} buffer protein calbindin D showed little conformational change in response to Ca^{2+}-binding. Troponin C contains 2 pairs EF-hands comprising N-terminal and C-terminal domains with each pair. The C-terminal domain of troponin C has high affinity and is therefore always occupied by Ca^{2+}. This domain is referred to the "structural domain" in contrast to the N-terminal domain which serves as a regulatory domain because it has low affinity and triggers Ca^{2+} signal leading to a specific response. Calmodulin shows a closed conformation in the Ca^{2+}-free state and an open conformation in the Ca^{2+}-bound stated. When bound Ca^{2+} calmodulin exposes its hydrophobic core (Yawaza, et al., 1987). This hydrophobic region is essential for the interaction with target proteins (Ikura, et al., 1992; Meador, et al., 1992, 1993). In plants, calmodulin and calcium-dependent protein kinase (CDPK) are the best known Ca^{2+} modulated proteins. Only few proteins have been reported to be regulated by calmodulin. Among those are NAD kinase, NTP kinase, glutamate decarboxylase, and Ca^{2+} ATPase both in plasma membrane and ER (Roberts and Harmon, 1992). CDPK is a large gene family of which activity is regulated

by Ca^{2+} but not by calmodulin. CDPK has been implicated in many physiological processes in plants (Roberts and Harmon, 1992). To understand physiological roles of CDPK in plants identifying individual isoforms and studying their biochemical properties are essential.

CHAPTER 2
CLONING OF cDNA, HETEROLOGOUS EXPRESSION, AND CHARACTERIZATION OF CDPKβ AND γ FROM SOYBEAN AND THEIR COMPARISON TO CDPKα

Introduction

CDPKs (Ca^{2+}-dependent protein kinases or calmodulin-like domain protein kinases) are a large family of protein kinases regulated by calcium but not by calmodulin (Hrabak et al., 1996; Roberts and Harmon, 1992). In plants, CDPK was first described (Harmon et al., 1987), highly purified (Putnam-Evans et al., 1990), and cloned (Harper et al., 1991) from soybean. CDPK is also characterized in protists. Two CDPK homologs were purified from *Paramecium* (Gunderson and Nelson, 1987; Son, et al., 1993) and molecular cloning of another from *Plasmodium* has been reported (Zhao, et al., 1993). The biochemical properties of CDPKs purified from soybean (Harmon, et al., 1987; Putnam-Evans et al., 1990) and *Paramecium* (CaPK-1 and -2) (Gunderson and Nelson, 1987; Son, et al., 1993) were similar in some aspects. Their molecular masses were 50 to 52 kDa, and free Ca^{2+} ($K_{0.5}$ of ~0.2 μM to 2 μM) was required for the enzyme activity and autophosphorylation. In contrast, these CDPKs have different substrate specificities. CaPK-1 and -2 have strong preference for casein over histone H1, but CDPK purified from soybean does not phosphorylate casein. Also, unlike the soybean enzyme, *Paramecium* CDPK required DTT (1,4-dithio-DL-threitol) for enzyme stability and could not use Mg^{2+}-GTP as a phosphate donor.

The catalytic domain of soybean CDPK (isoenzyme α) is related to that of Ca^{2+}/calmodulin dependent protein kinase type II (CaMK II). The C-terminal regulatory domain, like calmodulin, has four EF-hands. CDPKs also contain a short junction domain that links the catalytic and calmodulin-like domains and functions as an autoinhibitor (Harmon et al., 1994; Harper et al., 1994; Yoo and Harmon, 1996).

cDNAs encoding CDPKs have been cloned from carrot (Suen and Choi, 1991), *Arabidopsis* (Harper et al., 1991; Hong et al., 1996; Hrabak et al., 1996; Urao et al., 1994), rice (Breviario et al., 1995; Kawasaki et al., 1993), maize (Estruch et al., 1994; Takezawa, et al., 1996a), and mung bean (Botella et al., 1996). All of these CDPKs contain catalytic, junction, and calmodulin-like domains, and the amino acid sequence identity between CDPK isoforms ranges from 50% to 95%. Recently two protein kinases have been described that have catalytic domains related to those of CDPKs, but differ in their regulatory domains. The CDPK-like protein kinase from carrot has no predicted functional EF-hands in its carboxyl terminal domain (Lindzen and Choi, 1995). CCaMK from lily anthers has a regulatory domain that contains three EF-hands which are more similar to visinin than to calmodulin (Patil et al., 1995). Its activity, unlike that of CDPKs, is stimulated by calmodulin (Patil et al., 1995; Takezawa et al., 1996b).

In animals, cellular responses to calcium are brought about in part by two families of protein kinases; protein kinase C (PKC) and the calmodulin-dependent protein kinases. Activation of conventional PKCs (isoforms α, β, and γ) is dependent upon phosphatidyl serine, diacylglycerol, and the binding of Ca^{2+} to the C2 domain. Activation of the calmodulin-dependent protein kinases (phosphorylase kinase, myosin light chain kinases, and CaMKs I-IV) occurs through the binding of calcium to calmodulin. PKCs and most of the

calmodulin-dependent kinases are multifunctional enzymes that have broad substrate specificity (Dekker and Parker, 1994; Schulman, 1993). They are widely distributed in various tissues and cell types, and they are activated by a variety of external signals. An important question under active investigation is how different stimuli that act through calcium as the second messenger and a common set of calcium-regulated kinases are able to bring about specific responses. One answer suggested by recent data is that cellular signals direct the translocation and compartmentalization of specific PKC isotypes and thus target them to the locations of substrates involved in specific physiological functions (Mochly-Rosen, 1995).

In plants, intracellular free Ca^{2+} concentrations change in response to diverse stimuli such as growth regulators, red light, cold and salt stress, and fungal elicitors (Bush, 1995). To date no homologs of PKC and only one calmodulin-stimulated protein kinase (Patil et al., 1995; Takezawa et al., 1996b) have been characterized. It is likely that CDPKs are involved in mediating many of the diverse responses of plant cells to calcium. Specific roles for the CDPK isozymes in these responses could arise from differences in their biochemical properties, Ca^{2+} sensitivity, tissue distribution, compartmentalization, and substrate specificity. While the number of genes encoding CDPKs is growing, information regarding the properties of CDPK isoenzymes from a single plant species, and data linking CDPK isozymes to specific cellular responses are largely lacking. A single report made by Sheen (1996) showed that one CDPK isoform, but not others, is involved in expression of a reporter gene in response to stress.

To gain insight into how CDPK may play roles in transducing Ca^{2+} signals, I have undertaken a study of the biochemical properties of CDPK isoforms from soybean. cDNA clones encoding two new soybean CDPK

isoenzymes, CDPKβ and CDPKγ were isolated and sequenced. These were expressed in *Escherichia coli* (*E. coli*) and highly purified. The biochemical and kinetic properties of the new CDPKs and the previously described CDPKα, which is encoded by cDNA clone SK5 (Harper et al., 1991), were compared.

Experimental Procedures

Materials

A soybean plumule cDNA library was generously provided by E. Czarnecka-Varner and W. B. Gurley, University of Florida. A cDNA library made from soybean cell suspension culture was a gift from Drs. R. Tenhaken and C. Lamb, Salk institute. Membranes (Hybond-N^+ and -N) for plaque and RNA blotting were purchased from Amersham. Syntide-2 and oligonucleotides were synthesized by the Protein Chemistry and DNA Synthesis Core Laboratories, University of Florida. Autocamtide-2, skeletal and smooth muscle myosin light chain kinase substrates were purchased from BACHEM California. Protein kinase inhibitors were purchased from Calbiochem (H8, ML7, KN62, and staurosporine), Kamiya biochemical company (calphostin C, and K-252a), and from Seikagaku America, Inc. (KN93).

Plant Materials

Soybean cell suspension cultures (*Glycine max* L.) were maintained and prepared as described previously (Harmon et al., 1996). Soybean seeds (*Glycine max* L. cv. Cobb) were imbibed for 4 hours, disinfected in 10% Clorox for 10 minutes, rinsed several times with water, sown in perlite, and grown for 3 weeks in a green house.

Isolation of cDNA Clones, DNA Sequencing, and Sequence Analysis

Partial cDNAs encoding new CDPK isoforms were isolated by screening libraries with either cDNA probes or by PCR. A clone encoding CDPKβ was originally identified by screening a soybean plumule cDNA library with SK5 cDNA (Harper et al., 1991) as a probe. Plaque hybridization was performed at 65 °C overnight in 5x SSC (0.75 M NaCl, 75 mM Na_3 citrate), 5x Denhardt's solution (0.1% (w/v) BSA, 0.1% Ficoll, 0.1% PVP), 0.5% (w/v) SDS, and 20 μg/ml salmon sperm DNA according to the membrane manufacturer (Amersham). Filters were washed twice at room temperature for 10 minutes with 2x SSC, 0.1% (w/v) SDS, and once with 1x SSC, 0.1% SDS at 65 °C for 15 minutes. A 0.9 kb cDNA clone having a unique restriction pattern was identified. This clone was used to screen a cDNA library from soybean cell suspension culture at high stringency (wash with 0.1x SSC, 0.1% SDS at 65 °C for 10 minutes). CDPKγ was first identified by using a phage lysate of soybean nodule cDNA library as a template for PCR. Two degenerate oligonucleotide primers were used. The sense primer corresponded to the coding region for the conserved DLKPEN in subdomain VI-b of the protein kinase domain and was provided by Dr. J. Harper. The antisense primer, AS17 (5'-TCTAGAG-GATCCATC(ACT)A(GAT)(AT)GG(TC)TT(AG)TC(AT)G(GA)(TA)GC-3') was chosen from highly conserved sequence in the junction domains of CDPKs from soybean, *Arabidopsis* and carrot (Harper et al., 1991, 1993; Suen and Choi, 1991). The resulting 500 bp PCR product was subcloned in pUC 19 and sequenced to show that it encoded a new isoform. This partial cDNA clone was used as a specific probe for the isolation of full-length cDNA clones encoding CDPKγ by screening the cDNA library from soybean cell suspension culture cells.

DNA sequencing was performed manually by the dideoxy method (Sanger et al., 1977) using Sequenase version 2 (Amersham) according to the manufacturer's protocol with some modification. Plasmid DNA templates were first denatured in 1 M NaOH followed by neutralization in 1 M HCl (Hsiao, 1991). The denatured templates were annealed to a primer, labeled with [α-^{35}S] dATP, and the polymerization was terminated by adding ddNTP. The polymerization products were applied to glycerol-tolerant polyacrylamide gel in 1.43 M Tris, 0.46 M taurine, and 8.6 mM EDTA (ethylenediamine tetraacetic acid) (Pisa-Williamson and Fuller, 1992). The untreated gel was covered with plastic wrap, dried, and exposed to X-ray film for 16 to 48 hours.

DNA sequence analysis was aided by DNAStar for the Macintosh. GenBank Database searches were performed by the Blast Network Service provided by the National Center for Biotechnology Information through Interdisciplinary Center for Biotechnology Research at University of Florida.

RNA Analysis

Total RNA was isolated from soybean cell suspension cultures, eight-day-old seedlings, leaves, stems, roots, shoot-tips, and petioles of three-week-old soybean plants as described elsewhere (McCarty, 1986). RNA was blotted onto nitrocellulose membranes (Hybond-N, Amersham) by downward alkaline capillary transfer followed by electrophoresis in a denaturing formaldehyde/agarose gel as described by Chomczynski (1992). Hybridization was performed following standard protocols (Brown, 1992) in 50% formamide at 42 °C. Specific DNA probes for each isoform (5' untranslated regions from the cDNA clones encoding CDPKα and γ and the 3' untranslated region from cDNA clone encoding CDPKβ) were generated using PCR. After hybridization the blots were briefly washed several times in 2XSSC, 0.1% (w/v) SDS at room

temperature, twice for 10 minutes each in the same buffer, and finally twice in 1XSSC, 0.1% (w/v) SDS at 42 °C for 15 minutes. Due to high background, the blots hybridized with the CDPKβ-specific probe were washed further in 0.1XSSC, 0.1% (w/v) SDS at 65 °C for 10 minutes.

Construction of Plasmids

E. coli expression vector pGEX-KG (Guan and Dixon, 1991) was chosen to produce glutathione S-transferase (GST) -fusion proteins of full-length CDPKα, β, γ, and an N-terminal deletion mutant of CDPKγ containing amino acid residues 66-538, CDPKγ$_{(66-538)}$. pGST-CDPKα was generated by ligating the full-length cDNA insert cut from pHis1530 (Harmon et al., 1996) into pGEX-KG. For pGST-CDPKβ, first, the 5'-end of the cDNA encoding CDPKβ was amplified by PCR using 5'-GCTCTAGACCATATGCAGAAGCATGGT-3' (AS79) and 5'-GCCTTGTATCTGGACAACG-3' (AS62) as primers. The amplified DNA of 160 bp was purified from an agarose gel for enzyme digestion with *Xba*I and *Hind*III. Next, full-length cDNA clone of CDPKβ in pBlueScript was subjected to enzyme digestion with *Hind*III and *Xho*I, and 1.5 kbp fragment was isolated. These two fragments (160 bp and 1.5 kbp) were then subcloned into *Xba*I/*Xho*I double-digested pGEX-KG in one ligation reaction. For pGST-CDPKγ, a fragment of the 5'-end of cDNA clone encoding CDPKγ was amplified by PCR using 5'-GCTCTAGACCATATGGTTACAGAC-ATGCT-3' (AS77) and 5'-GGAATTCTTAAAGTGTGTGGAACTGCT-3' (AS75) as primers. The fragment of 115 bp was purified from an agarose gel and was digested with *Xba*I and *Sph*I and ligated into the *Sph*I-digested full-length cDNA clone of CDPKγ (1.5 kbp), and then the resulting 1.6 kbp fragment was subjected to *Xba*I digestion in order to subclone into *Xba*I-digested pGEX-KG. To make the N-terminal deletion mutant of CDPKγ, A DNA fragment (200

bp) amplified using the primers 5'-GCTCTAGACCATATGGGTGTTAGGCA-AGAC-3' (AS78) and 5'-TAATCCATGGGTGCTCAA-3' (AS44) was digested with *Xba*I and *Sst*I, and subcloned into *Xba*I-digested pGEX-KG together with *Sst*I/*Xba*I double-digested cDNA clone of CDPKγ (1.2 kbp). Constructs expressing active recombinant proteins were selected and confirmed to be error-free by DNA sequencing. The PCR primers AS77-79 were designed to contain *Xba*I and *Nde*I restriction sites separated by one base pair to keep the correct reading frames in any subcloning vectors using either sites. The two bases at the 5' end of these primers were added for the efficient digestion with *Xba*I.

In attempts to produce polyclonal antibodies specific to CDPKβ and γ, N-terminal sequences of each isoform were chosen to produce GST-fusion proteins. Because the N-terminal domain of CDPKβ was short (23 amino acid residues), part of catalytic domain was included. pGST-CDPKβ was digested with *Xba*I and *Hind*III to generate a 230 bp DNA fragment encoding 60 amino acid residues of CDPKβ N-terminus and subcloned into *Xba*I/*Hind*III double-digested pGEX-KG. The fidelity was confirmed by expression and DNA sequencing. Since the N-terminal domain of CDPKγ contains 83 unique amino acid residues, this region seemed an excellent choice for raising specific antibodies. The fusion protein containing this region was created as follows. As mentioned above, pGST-CDPKγ was subcloned into pGEX-KG through *Xba*I sites. The insert of pGST-CDPKγ contains three *Xmn*I sites and the vector contains one *Xmn*I site. Digestion with *Xmn*I generates blunt ended DNA. Because the most 5' side *Xmn*I site in the insert was located at around amino acid residue 80 of CDPKγ, this restriction enzyme was utilized together with *Xba*I to produce a 240 bp DNA fragment. Other small sized fragments (30, 320, 630, 800 bp) resulting from the double digestion were separated from

this fragment in a 2% agarose gel. Due to the limited number of cloning sites and their orientation in native pGEX-KG, the following modifications were made. pGST-CDPKβ was first digested with *Xho*I to cut 3'-end of the insert followed by blunt-ending by Klenow enzyme treatment for 15 minutes at 25 °C and for another 15 minutes at 75 °C to inactivate the enzyme and then digested with *Xba*I to cut out the insert from the rest of the plasmid. The resulting pGEX-KG vector contained the partial multicloning sites originating from pBlueScript, and was ligated with the purified 240 bp fragment described above. The transformants were screened by digesting plasmids with *Xba*I and *Hind*III which cuts the right side of 3' end of the 240 bp insert prior to expression.

Expression and Purification of GST-Fusion Proteins

A colony of transformed *E. coli* cells (PR745) was grown overnight at 37 °C in 2 ml LB/ampicillin (0.1 mg/ml) and transferred to 500x dilution into M9TB/ampicilin (Studier et al., 1990) and further cultured until OD_{600} ~ 0.5. Expression of recombinant protein was induced by adding IPTG (isopropyl 1-thio-β-D-galactopyranoside) to 0.4 mM at room temperature. Cells expressing CDPKβ were collected and resuspended in lysis buffer containing 1 mM PMSF (phenylmethylsulphonyl fluoride), 10 μg/ml leupeptin, 20 μg/ml aprotinin, 1 mM DTT, 50 mM Tris, pH 7.5, and 150 mM NaCl and centirifuged after sonication (Yoo and Harmon, 1996). The supernatant was loaded onto a glutathione-agarose column equilibrated with 50 mM Tris, pH 7.5, 150 mM NaCl, and 1 mM DTT, and washed with the same buffer. Protein was eluted with 50 mM Tris, pH 8.0, 1 mM DTT, and 10 mM glutathione. Fractions containing kinase activity were pooled, and loaded onto Mono-Q equilibrated in 20 mM Tris, pH 7.2, 1 mM $CaCl_2$, 2% (w/v) betaine, and 1 mM DTT. CDPKβ

was eluted with a gradient of 0-0.5 M NaCl in equilibration buffer. Fractions containing kinase activity were pooled again, and further purified by another Mono-Q chromatography in the absence of calcium but in the presence of 2.5 mM EDTA. The procedure for purification of CDPKα, γ, and $\gamma_{(66-538)}$ was as follows. Cell lysis and affinity chromatography were performed in buffer A (50 mM Tris, pH 7.5, 150 mM NaCl, 10 mM EDTA, and 2 mM DTT). The debris in the lysed cells was pelleted by centrifugation, and the supernatant was loaded onto a glutathione-agarose column. The column was washed with buffer A and protein was eluted with buffer B (50 mM Tris, pH 8.0, 10 mM glutathione, 10 mM EDTA, and 2 mM DTT). Fractions containing kinase activity were pooled and loaded onto a column of Mono-Q (Pharmacia) equilibrated in buffer C (20 mM Tris, pH 8.0, 2.5 mM EDTA, 5% (w/v) betaine, 2 mM DTT). Enzymes were eluted with a gradient of 0-0.5 M KCl in buffer C. Purified recombinant CDPKα, and β were dialyzed against 20 mM Tris, pH 8.0, 14.4 mM 2-mercaptoethanol and stored at -80 °C in 50% glycerol (v/v). CDPKγ was labile to freeze/thaw, so eluted pure fractions were stored at 4 °C.

The N-terminal domains of CDPKβ and γ expressed as GST fusion proteins in *E. coli* were prepared and affinity purified on a glutathione-agarose column in a same manner as described above. The protein eluates of both constructs were came off during the wash step when loaded onto the Mono-Q column. SDS-PAGE of the fractions showed that some higher and smaller molecular weight contaminants were separated from main protein bands of expected size for the constructs. Therefore, no further purification was undertaken.

Concentrations of recombinant CDPKs were determined according to the method of Bradford (Bradford, 1976) using Bio-Rad dye-binding assay.

Protein Kinase Assays

Enzyme activity assays were performed by a modification of the procedure described previously (Harmon et al., 1996). The reaction mixture contained 50 mM HEPES, pH 7.2, 10 mM $MgCl_2$, 1 mM EGTA, 1.1 mM $CaCl_2$, 2 mM DTT, 0.1 mg/ml BSA, the indicated amount of substrates, 5 nM recombinant CDPK, and 60 μM ATP (~500 cpm/pmol). The concentrations of synthetic peptides (syntide-2, autocamtide-2, and skeletal and smooth muscle myosin light chain kinase substrates) and histone IIIS in the assays were 100 μM and 0.5 mg/ml, respectively. Kinetic parameters (apparent K_m and V_{max}) were determined from double reciprocal plots. Concentrations of synthetic peptides were determined from amino acid composition analyses carried out by Protein Chemistry Core Facility, University of Florida. Each kinetic parameter was determined by averaging at least four independent assay results.

Determination of IC_{50} Values with Protein Kinase Inhibitors

Stocks of protein kinase inhibitors (1 mg/ml) were prepared in DMSO. Various concentrations of inhibitors in the enzyme activity assay were achieved by diluting stock solutions and the final dilutions were made in 0.1% (v/v) DMSO. Control assays (total 50 μl in each reaction) were done in the presence of 5 μl of 0.1% (v/v) DMSO. The enzyme activity assay mixture was the same as described above and contained 100 μM syntide-2 as peptide substrate. IC_{50} values were obtained by averaging enzyme activities from at least four determinations.

Preparation of His_6-CDPK Constructs and Purification of Expressed Proteins

Recombinant His_6-CDPK constructs were generated by digesting pGST-$CDPK\gamma_{(66-538)}$ with *Nde*I and *Xho*I and subsequent subcloning into *Nde*I/*Xho*I double-digested pET-15b. The transformants were screened for the right insert size by digesting the plasmids with *Nde*I and *Xho*I. His_6-$CDPK\gamma_{(66-538)}$ was purified as follows. The expression of transformed cells and chromatography using a nickel chelation column were carried out following the procedures described elsewhere (Yoo, et al., 1996). The eluates were dialyzed against buffer A containing 20 mM Tris, pH 8.0, 2.5 mM EDTA, and 100 mM KCl and then loaded onto Mono-Q column equilibrated with buffer A. After washing in buffer A, the protein was eluted with a gradient from 0.1-0.5 M KCl in buffer A. Fractions were analyzed by SDS-PAGE and pooled for further purification by phenyl-Superose reverse phase chromatography. Equilibration buffer contained 20 mM Tris, pH 8.0, 2.5 mM EDTA, and 1.5 M ammonium sulfate. Following loadings of the pooled fractions from the Mono-Q column onto a phenyl-Sepharose column, and washing of the resin with equilibration buffer, the protein was eluted with a reverse gradient of 100-0%. Highly purified His_6-CDPKγ resulting from this chromatography was concentrated using a Centricon-30 (Amicon) filter.

Preparation of Microsomal Membrane

Cultured soybean cells were homogenated in Buffer A containing 2.5% sucrose, 25 mM Tris, pH 7.8, and 0.5 mM $CaCl_2$ using glass-glass homogenizer and passed through cheese cloth. The filtrate was centrifuged at 8,000g for 15 minutes and the supernatant was ultracentrifuged at 50,000g for 30 minutes. The supernatant was saved and the pellet was dissolved in buffer A without

sucrose (buffer B) and centrifuged at 5,000g for 30 minutes. The pellet was dissolved in buffer B and the protein amount was measured by Bradford assay (Bradford, 1976).

Other Procedures

SDS-PAGE was performed according to the method of Laemmli (Laemmli, 1970). Electroblotting was performed in a buffer containing 25 mM Tris, 192 mM glycine, pH 8.3, and 20% (v/v) methanol for at least 2 hours. Blotted nitrocellulose membranes were blocked in 5% nonfat milk dissolved in TBS (20 mM Tris, pH 7.5 and 500 mM NaCl) for 30 minutes and incubated with the first antibody for 1-2 hours. The blots were washed in TBS containing 0.05% Tween-20 several times followed by incubation with the second antibody for 30-60 minutes. The blots were washed again and subjected to color development in a solution containing 0.15 M $NaHCO_3$, pH 9.6, 4 mM $MgCl_2$, 0.1 mg/ml NBT, and 50 ng/ml BCIP. Digital images of polyacrylamide gels and nitrocellulose blots were obtained as previously described (Harmon et al., 1994). Oligonucleotides were synthesized at the Interdisciplinary Center for Biotechnology Research DNA Synthesis Lab, University of Florida.

Results

cDNA Clones that Encode Two New CDPK Isoforms from Soybean

The nucleotide and deduced amino acid sequences of cDNA clones encoding CDPK isoforms, CDPKβ and CDPKγ, are shown in Figure 2-1. The cDNA encoding CDPKβ contains an open reading frame 1470 nucleotides in length without an in-frame stop codon at the 5' end. The size of the

Figure 2-1. Nucleotide and deduced amino acid sequences of two new CDPK isoenzmes from soybean. Nucleotide sequences are shown in the numbered rows and the predicted amino acid sequences (single letter code) of the longest open reading frames are shown beneath the corresponding cDNA sequence. Possible poly (A) signals are underlined.
a) CDPKβ; b) CDPKγ.

A. CDPKβ

```
TCGGCTACCTTGGCTATAGTTTACGTGTTGAGAGGGAGAGAGAGAGGAAACAAAATGC   60
                                                        M  Q

AGAAGCATGGTTTTGCATCAAAGGGCAACGTGTTGCCGTATGAAACGGCGAGGCTAAGGG  120
  K  H  G  F  A  S  K  K  N  V  L  P  Y  Q  T  A  K  L  K  D

ACCACTACGTTCTGGGGAAGAAGGTGGGGCAAGGGCAATTCGGGACGACGTAGCTGTGCA  180
  H  Y  V  L  G  K  K  L  G  Q  G  Q  F  G  T  T  Y  L  C  T

CCCACAAGGTGACGGGGAAGCTCTACGCGTGCAAATCGATCCCGAAGAGGAAGGTTATGT  240
  H  K  V  T  G  K  L  Y  A  C  K  S  I  P  K  R  K  L  M  C

GCCAGGAGGATTACGATGATGTGTGGAGGGAGATTCAGATCATGCACCATTTGTCGGAGG  300
  Q  E  D  Y  D  D  V  W  K  E  I  Q  I  M  H  H  L  S  E  H

ATCCAAACGTTGTCCAGATACAAGGCACGTACGAGGATTGCGTGTTCGTGCACCTTGTCA  360
  P  N  V  V  Q  I  Q  G  T  Y  E  D  S  V  F  V  H  L  V  M

TGGAACTATGTGCCGGCGGGGAGCTTTTCGACAGGATGATTCAGAAGGGGCATTACAGCG  420
  E  L  C  A  G  G  E  L  F  D  K  I  I  Q  K  G  H  Y  S  E

AGAGAGAGGCTGCCAAGTTGATAAAGACCATTGTTGGGGTGGTGGAGGCGTGCCACTCTG  480
  R  E  A  A  K  L  I  K  T  I  V  G  V  V  E  A  C  H  S  L

TTGGGGTCATGCATAGGGATCTCAAGCCTGAGAATTTCTTGTTTGATACCCCTGGCGAAC  540
  G  V  M  H  R  D  L  K  P  E  N  F  L  F  D  T  P  G  E  D

ATGCCCAGATGAAGGCCACCGATTTTGGCCTCTCTGTCATTGTACAAGCCAGACAACCCT  600
  A  Q  M  K  A  T  D  F  G  L  S  V  I  L  Q  A  K  Q  A  F

TTCATGATGTAGTAGGAAGTGCCTACTATGTTGCCCCAGAGGTGTTGTGCAAGCAATATG  660
  H  D  V  V  G  S  P  Y  Y  V  A  P  E  V  L  C  K  Q  Y  G

GACCTGAAGTGCACGTATGGAGTGCTGGTGTTATCCTATACATCTTACTGAGTGGGGTGG  720
  P  E  V  D  V  W  S  A  G  V  I  L  Y  I  L  L  S  G  V  P

CACCTTTCTGGGCTGAAACCGAACCAGGAATTTTCAGACAGATTTTAAATGGAGATGTTG  780
  P  F  W  A  E  T  E  A  G  I  F  K  Q  I  L  N  G  D  L  D

ATTTTGTTTCTGAACCGTGGCCAAGTATCTCACAAAATGCTAAAGAATTGGTAAAACAGA  840
  F  V  S  E  P  W  P  S  I  S  E  N  A  K  E  L  V  K  Q  M

TGTTGGATAGGGACCCTAAGAAAAGAATTTGTGCTCATGAACTTTTATGTAACCCTTGGG  900
  L  D  R  D  P  K  K  R  I  S  A  K  E  V  L  C  N  P  W  V

TTGTTGATGACATTGCACCTGACAAACCTCTCGACTCTGCTGTTTTGACACGCCTAAAGC  960
  V  D  D  I  A  P  D  K  P  L  D  S  A  V  L  T  R  L  K  H

ATTTCTGAGCAATGAATAAACTTAAGAAGATGGCATTACGGGTCATAGCAGAGAGGGTTT 1020
  F  S  A  M  N  K  L  K  K  M  A  L  K  V  I  A  E  R  L  S

CAGAGGAAGAAATACGTGGATTGAAAGAGTTGTTTAAAATGATTGACACAGAGAATAGTG 1080
  R  E  E  I  G  G  L  K  E  L  F  K  M  I  D  T  D  N  S  G

GGACAATAACTTTTGAGGAACTGAAGGAGGGGTTGAAAAGTGTGGGCTCTAATGTCATGG 1140
  T  I  T  F  E  E  L  K  E  G  L  K  S  V  G  S  N  L  M  E

AATGTGAAATTAAATCACTTATGGAAGCGGCTGATATAGAGAAGAATGGATCAATAGACT 1200
  S  E  I  K  S  L  M  E  A  A  D  I  D  N  N  G  S  I  D  Y

ATGGTGAATTTGTTGCTGCTACACTGGACTTGAATAAGATGGAAAGAGAGGAGAATTTGG 1260
  G  E  F  L  A  A  T  L  H  L  N  K  M  E  R  E  E  N  L  V

TTGCTGCTTTCGCCTATTTTGATAAAGATGGTAGTGGTTAGATCACCATTGACGAGCTTC 1320
  A  A  F  A  Y  F  D  K  D  G  S  G  Y  I  T  I  D  E  L  Q

AACAGGCTTGTAAGGACTTCAGCCTAGGTGATGTGCATGTGGATGAGATGATCAAAGAGA 1380
  Q  A  C  K  D  F  S  L  G  D  V  H  L  D  E  M  I  K  E  I

TTGATGAAGATAATGATGGGAGGATTGATTATGCGGAGTTTGCAGCAATGATGAAAAAGG 1440
  D  Q  D  N  D  G  K  I  D  Y  A  E  F  A  A  M  M  K  K  G

GTGATGCAAATATGGGTAGAAGCAGAACCATGAAAGGCAATTTGAACTTGAATATTGCAG 1500
  D  P  N  M  G  R  S  R  T  M  K  G  N  L  N  F  N  I  A  D

ATGCATTTGGAATGAAAGACTCTTCTTGATATGCTACCTGAATTCTTTTGTACAGTTGGA 1560
  A  F  G  M  K  D  S  S  *

TGCATGATGGTAGAAGAAAAATCATTGTCAAAATTTCTTATGCGCGTGGTTATAATGTGT 1620
TTTGTATTGGAACTTTGTTTACTTTCTCAAGCTATGCTTTAATCCCTTAAGTTTAGAAAC 1680
CTTCAGTGAAGTTGAATATATTCAGCATATATATGATATAAATTGGCTTGAATGAAAAAA 1740
AAAAAAAAAAAAA
```

B. CDPKγ

```
GAACTGCAGCTTATGTAGTACTAGCATTTATCCCTTTTTTTTTTTTTATACATTCTTTCT   60
CTTTCACCCTCTTTGAAGGCTCCACTTTTATAATTGCTTGTAATTTCTTCATCAAAGTAT  120
CAAACTTGTCTTCTGTCTGTGTCCTTTTACTTGCTGTGCATATATGATATATTGTTGCAA  180
AATCCTCATCTAAGGGTTAATTTTGTTCCGGACAAGTTTTTATTATTATTATTATTATTT  240
TTTAAATTTCAGAATGATTTACTTATAGGTTTCTGATCCAAGGTTTAAGATGTTATAGTT  300
TGCTTGAATTCTCATGTGGGTTCAACCTGTTTGATGTTTTGACTGAATCAGTTTGAAATA  360
GTGGTGATTATGTGATACCAAACATAAAAGATTGAGCTGTTTTTGTTGTTTCTTTGTCAC  420
AAGACTTGGATTTTGAGAATGTGGAGACATGGGTTGTCGTGCAAGTAAAGAGTGAGACCC  480
GCAACACAATGGTTACAGACATGCTGGGACTGGTGGTGTGCACAACGAAGAAGACTGATG  540
        M  V  T  D  M  L  G  L  V  V  C  T  T  K  K  T  H  E

AAGCTTTGGTGAACCAATCAAGAGCACCTGCAAATGAAGCTTATGATTTACATGAAAAGC  600
  P  L  V  N  Q  S  K  A  P  A  N  Q  P  Y  E  L  H  E  K  H

ATGCTGCATCCACTGCTGAGACAGTGCCTGAAAATATGCCATGGAAGCCTGCTGGTGCAG  660
  A  A  S  T  A  Q  T  V  P  Q  N  M  P  W  K  P  P  G  P  A

CCCTTAGTGCTAAACCTGTTGTTGGTGTTAGGCAAGACACAATTGTTGGGAAGCAGTTTG  720
  L  S  P  K  P  V  V  G  V  K  Q  D  T  I  L  G  K  Q  P  E

AGCATGTGAAGCAGTTCCACACACTTGGGAAGCAGTTGGGTAGAGGGCAATTTCGTGTGA  780
  D  V  K  Q  F  H  T  L  G  K  E  L  G  K  G  Q  F  G  V  T

CATATCTTTGCACTGAGAATTCGACCGGATTGCAGTATGCCTGCAAGTGCATTTCCAAGA  840
  Y  L  C  T  E  N  S  T  G  L  Q  Y  A  C  K  S  I  E  K  K

GGAAACTTGCGAGCAAATCTGATAAGGAGGACATAAAGAGGGAGATTCAGATTATGCAGC  900
  K  L  A  S  K  S  D  K  E  D  I  K  K  E  I  Q  I  M  Q  H

ATTTGAGTGGTGAAGCGAACATTGTTGAGTTCAAAGGGCCTTATGAGGATAGGAGCTCAG  960
  L  S  G  Q  P  N  I  V  E  F  K  G  A  Y  E  D  K  S  S  V

TTGATGTTGTGATGCAGCTGTGTGCAGGTGGGCAAGTTTTTGATAGGATTATTGCGAAGC 1020
  H  V  V  M  E  L  C  A  G  G  E  L  F  D  R  I  I  A  K  G

GGCATTACAGTGAGAAGGCTGCTGCTTCAATTTGCAGACAAATTGTAAATGTTGTTCATA 1080
  H  Y  S  E  K  A  A  A  S  I  C  K  Q  I  V  N  V  V  H  I

TCTGTGATTTCATGGGTGTGATGCATAGGGATGTGAAACCAGAGAATTTTTTGCTATCTA 1140
  C  E  F  M  G  V  M  E  K  D  L  K  P  E  N  F  L  L  S  S

GTAGGCACGAAAATGCACTTGTCAAGGCAACCGATTTTGGCTTGTCAGTTTTCATTGAAG 1200
  K  D  E  N  A  L  L  K  A  T  D  F  G  L  S  V  P  I  E  E

AAGGAAAGGTATATGGGGATATAGTTGGTAGTGCTTACTATGTTGCTGCTGAAGTTCTGC 1260
  G  K  V  Y  K  D  I  V  G  S  A  Y  Y  V  A  P  E  V  L  E

GGCGCAGATGTGGGAAGGAAATAGATATATGGAGTGCAGGAGTCATATTGTATATCTTAC 1320
  K  K  C  G  K  E  I  D  I  N  S  A  G  V  I  L  Y  I  L  L

TTAGTGGAGTCCCTGCATTTTGGCCTGAGACTGAGAAGGGAATATTTGATGCCATATTGG 1380
  E  G  V  P  P  F  W  A  E  T  E  K  G  I  F  D  A  I  L  E

AAGGTGACATTGATTTTGAAAGTCAACCATGGCCTAACATCTCAGACAGTGCCAAGCATG 1440
  G  H  I  D  F  E  S  Q  P  W  P  N  I  S  D  S  A  K  D  L

TTGTTCGTAAGATGCTTATAGAGGATGCAAAGAAACGCATTACCTCTGCTGAAGTTCTTG 1500
  V  K  K  M  L  I  Q  D  P  K  K  K  I  T  S  A  Q  V  L  E

AGCACCCATGGATTAAAGATGGAAATGCTTCAGAGAAGCCGATAGACAGTGCAGTCCTTT 1560
  H  P  W  I  K  D  G  N  A  S  D  K  P  I  D  E  A  V  L  S

CCAGAATGAAGCAATTTAGAGCAATGAATAAGCTAAAGAAACTTGCCTTGAAGGTCATTG 1620
  K  M  K  Q  F  R  A  M  N  K  L  K  K  L  A  L  K  V  I  A

CTGAGAATATGTGTGCAGAAGAGATCCAAGGTTTGAAGCCAATGTTTACAAATATGGACA 1680
  E  N  M  S  A  E  E  I  Q  G  L  K  A  M  F  T  N  M  D  T

CTGACAAGAGTGGTACAATGACCTATGAAGAAGTTAAGTGAGGATTGCATAGACTTGGCT 1740
  D  K  S  G  T  I  T  Y  E  E  L  K  S  G  L  H  E  L  G  S

CAAAGCTTACAGAGGCTGAAGTGAAGCAAGTTATGCAAGCTGCTGATGTAGATGGAAATG 1800
  K  L  T  E  A  E  V  K  Q  L  M  E  A  A  D  V  D  G  N  G
```

Figure 2-1 continued

```
GCTCAATTGACTACATAGAATTCATCACTGCTACAATGCATAGACACAAATTAGAAAGAG 1860
  S  I  D  Y  I  E  F  I  T  A  T  M  H  R  H  K  L  E  R  D

ATGACCAACTTTTCAAGGCCTTCCAATATTTTGATAAAGACAACAGTGGGTTTATTACAA 1920
  D  Q  L  F  K  A  F  Q  Y  F  D  R  D  N  S  G  F  I  T  R

GAGATGAATTGGAATCAGCCATGAAAGAATATGGTATGGGTGATGATGCAACAATCAAGG 1980
  D  E  L  E  S  A  M  K  E  Y  G  M  G  D  D  A  T  I  K  E

AAATCATATCTGAAGTTGATACAATTATATCTGAAGTGGATACAGATCATGATGGTAGAA 2040
  I  I  S  E  V  D  T  I  I  S  E  V  D  T  D  H  D  G  R  I

TGAACTATGAAGAATTCTCTGCGATGATGAAGAGTGGGAACCAACAACAAGGCAAGCTAT 2100
  N  Y  E  E  F  S  A  M  M  K  S  G  N  Q  Q  Q  G  K  L  F

TCTAAATCACACCATGTAACCAGTCTGAGGGCCTGGTATCCAAAACCCCTGTAGACAAAA 2160
GGTTTATACCTGTGAGAAGTACTTCCAAACAGTTTGTATGACTCATAGGCATGTAAAGCT 2220
TCTATTTAGAATGTTTTTGAGTTACTATGCTGCTCATGACTGTTAGTGAACTCCCTTTTT 2280
TTCATTTCTGGGACCTTTTTTTGCTTTTCACTGAGATTCCATTTCCGAGKCCTCTTTGAA 2340
TTTGTTTTGGACACAAAGTATAAACTCACATAGAAAAAGGTGATATCTCAATAAGATTAT 2400
ACAAGTTTTtGCGCTTGAAAAAAAAAAAAAAAAAAAA
```

Figure 2-1 continued

transcript observed on an RNA blot probed with CDPKβ-specific sequence is approximately 1.8 kb (data not shown) which is similar to the size of the cloned DNA (1.75 kb). Attempts to isolate longer cDNA clones from the cDNA library yielded only clones of the same length, and attempts to isolate clones containing additional 5′ sequence by anchored PCR were not successful. Taken together, these results suggest that the isolated cDNA encodes full-length CDPKβ. The predicted protein contains 490 amino acid residues and has a molecular mass of 55 kD.

Four cDNA clones that hybridized to unique DNA probe generated from PCR using nodule-enriched cDNA library lysate as template were purified and sequenced. Three of the overlapping clones had inserts ranging 2.1 to 2.4 kb, and the fourth clone was 3.2 kb. Figure 2-1B shows the sequence of the 2.4 kb clone. This clone and the two smaller ones contained an identical open reading frame, 1,614 nucleotides in length, that encodes a protein kinase 538 residues in length having a predicted molecular mass of 60 kD. This protein was named CDPKγ. The 3.2 kb clone encoded a predicted protein almost identical to CDPKγ, but also contained an unidentified upstream open reading frame that was presumed to have arisen as a cloning artifact.

Deduced Amino Acid Sequences of Soybean CDPK Isoforms

CDPKβ and CDPKγ share 76% and 58% overall amino acid sequence identity, respectively, with CDPKα. CDPKβ shows highest identity to AtCPK4 (Hrabak et al., 1996) and Atcdpk2 (Urao et al., 1994) (80% and 79%, respectively). CDPKγ shows highest identity to OSCPK2 (Breviario et al., 1995) and AtCPK9 (Hrabak et al., 1996), (77% and 74%, respectively), and to the

partial sequence of carrot CDPK (Suen and Choi, 1991) (82%, in the 425 residue overlap).

The amino terminal domains of CDPKα, CDPKβ, and CDPKγ are 33, 23, and 83 residues in length, respectively. The sequences of these domains match no known proteins and they do not contain any known sequence motifs. There is little similarity in sequence among the amino terminal domains (10-185 residues in length) of 24 CDPKs in the GenBank database, except for a high proportion of hydrophilic residues with more being basic than acidic. The amino terminal domain of CDPKγ is about 10% proline, as are most CDPK amino termini that are greater than 74 amino residues in length.

Figure 2-2 shows the amino acid sequence alignment and consensus sequences of the catalytic (*Panel* A), junction (*Panel* B), and calmodulin-like domains (*Panel* C) of CDPKs from different plant species. All kinase subdomains and four EF-hands are highly conserved among them. CDPKγ contains additional eight amino acid residues between the third and fourth EF-hands in the calmodulin-like domain which is the longest insert in that region compared to the others. Alignment of the sequences by the CLUSTAL algorithm shows that sequences flanking the fourth EF-hand are the least conserved regions (Figure 2-2C). These regions of low conservation may imply functional differences.

RNA Expression Pattern of CDPK Isoforms

Total RNA from different organs of soybean plants or from suspension-cultured cells was blot hybridized with DNA probes specific for genes encoding each of CDPK isoenzymes (Figure 2-3). CDPKα, β, and γ transcripts were expressed in roots, leaves, petioles, stems, shoot tips of three-

Figure 2-2. Alignment of amino acid residues of soybean CDPK isoforms with known CDPKs from other plants. Multisequence alignment was performed using sequence analysis software CLUSTAL provided by Genetics Computer Group. Gaps introduced for optimal alignment are indicated by dashes. The three functional domains are compared separately and the positions of amino acid residues are indicated in parentheses at the beginning of each panel. Residues and symbols in the consensus sequence are: capital letters, absolutely conserved residues; lower case letters, residues conserved in >70% of the isoforms; -, acidic residues; +, basic residues; #, aliphatic residues; $, serine or threonine. The sequences are CDPKα (Harper et al., 1991), β, and γ from soybean;VrCDPK (Botella et al., 1996) from mung bean (*Vigna radiata*) ; AtCPK1 to AtCPK8 (Hrabak et al., 1996) and ATCDPK1 and 2 (Urao et al., 1994) from *Arabidopsis* ; SPK (Kawasaki et al., 1993) and OsCPK 2 and 11 (Breviario et al., 1995) from rice; ZmCPK (Estruch et al., 1994) from maize; DcCDPK (Suen and Choi, 1991) from carrot.
a) Catalytic domains. The twelve conserved kinase catalytic subdomains are designated by Roman numerals; b) Junction Domains; c) Calmodulin-like Domains. Each Ca^{2+}-coordinating loop of the four EF-hands is indicated as I to IV.

A. Catalytic Domaiain

```
                          I                           II                    III                  IV                      V
Consensus        y~lg+~lG~gqFG~Tylct~~~•g~~yACKsI~krkL~~~~D~eDvrREiqIm~hl~~~~n#v~#~~~yED~~~Vh#vME1C~GGELFdRI~~+Ghy Er

CDPKα    (34~295)  YEVGRKLGQGQFGTTFECTRRASGGKFACKSIPKRKLLCKEDYEDVWREIQIMHHLSEHANVVRIEGTYEDSTAVHLVMELCEGGELFDRIVQKGHYSER
CDPKβ    (24~285)  YVLGKKLGQGQFGTTYLCTHKVTGKLYACKSIPKRKLMCQEDYDDVWREIQIMHHLSEHPNVVQIQGTYEDSVFVHLVMELCAGGELFDRIIQKGHYSER
CDPKγ    (84~344)  HTLGKELGRGQFGVTYLCTENSTGLQYACKSISKRKLASKSDKEDIKREIQIMQHLSGQPNIVEFKGAYEDRSSVHVVMELCAGGELFDRIIAKGHYSEK
SPK      (73~334)  YTLGRELGQGQFGKTYLCTEISTGCQYACKTILKSNLRCVSDIEDVRREIQIMHHLSGQKNIVTIKDTYEDEQAVHIVMELCAGGELFSKIQKRGHYSER
DsCPK2   (85~346)  YSLGKELGRGQFGVTYLCTEIASGKQYACKSISKRKLVSKADKEDIRREIQIMQHLSGQQNIVEFRGAYEDKSNVHVVMELCAGGELFDRIIAKGHYSER
QsCPK11  (79~340)  YIIGRKLGQAQFGTTYLCTEINTGCEYACKTIPKRKLITKEDVEDVRREIQIMHHLSGHKNVVAIKDVYEDGQAVHIVMELCAGGELFDRIQEKGHYSER
ZmCDPK   (93~354)  YSMGKELGRGQFGVTHLCTHRTSGEKLACKTIAKRKLAAREDVDDVRREVQIMHHLSGQPNVVGLRGAYEDKQSVHLVMELCAGGELFDRIIARGQYTER
Atcdpk1  (11~272)  YILGRELGRGEFGITYLCTDRETHEALACKSISKRKLRTAVDIEDVRREVAIMSTLPEHPNVVKLKASYEDNENVHLVMELCEGGELFDRIVARGHYTER
Atcdpk2  (26~287)  YLLGKKLGQGQFGTTYLCTEKSTSANYACKSIPKRKLVCREDYEDVWREIQIMHHLSEHPNVVRIKGTYEDSVFVHIVMEVCEGGELFDRIVSKGHFSER
CPK1    (150~411)  YSLGRKLGQGQFGTTFLCVEKTTGKEFACKSIAKRKLLTDEDVEDVRREIQIMHHLAGHPNVISIKGAYEDVVAVHLVMECCAGGELFDRIIQRGHYTER
CPK2    (186~447)  YSLGRKLGQGQFGTTFLCLEKGTGNEYACKSISKRKLLTDEDVEDVRREIQIMHHLAGHPNVISIKGAYEDVVAVHLVMELCSGGELFDRIIQRGHYTER
CPK3     (77~336)  YEFGRELGRGQFGVTYLVTHKETKQQVACKSIPTRRLVHKDDIEDVRREVQIMHHLSGHRNIVDLKGAYEDRHSVNLIMELCEGGELFDRIISKGLYSER
CPK4     (25~286)  YLLGKKLGQGQFGTTYLCTEKSSSANYACKSIPKRKLVCREDYEDVWREIQIMHHLSEHPNVVRIKGTYEDSVFVHIVMEVCEGGELFDRIVSKGCFSER
CPK5     (97~357)  YTLSRKLGQGQFGTTYLCTEIASGVDYACKSISKRKLISKEDVEDVRREIQIMHHLAGHGSIVTIKGAYEDSLYVHIVMELCAGGELFDRIIQRGHYSER
CPK6     (85~346)  YTLSRKLGQGQFGTTYLCTDIATGVDYACKSISKRKLISKEDVEDVRREIQIMHHLAGHKNIVTIKGAYEDPLYVHIVMELCAGGELFDRIIHRGHYSER
CPK7     (59~321)  YDLGREVGRGEFGITYLCTDKETGEKYACKSISKKKLRTAVDIEDVRREVEIMKHMPKHPNVVSLKDSFEDDDAVHIVMELCEGGELFDRIVARGHYTER
CPK8     (57~318)  YDLGREVGRGEFGITYLCTDIKTGEKYACKSISKKKLRTAVDIEDVRREVEIMKHMPRHPNIVSLKDAFEDDDAVHIVMEXCEGGELFDRIVARGHYTER
CPK9     (91~352)  YTLGKELGRGQFGVTYLCTENSTGKKYACKSISKKKLVTKADKDDMRREIQIMQHLSGQPNIVEFKGAYEDEKAVNLVMELCAGGELFDRIIAKGHYTER
DcCDPK   ( 1~235)  ••••••••••••••••••••••••ACKSILKRKLVSKNDKEDIKREIQILQHLSGQPNIVEFKGVFEDRQSVHLVMELCAGGELFDRIIAQGHYSER


                VI~a                   VI-b                    VII                  VIII               IX
Cons.     ~Aa~#~+~i#~vv~~CH~~GVmHRD1KPENFL~~~~~~~~~lKa~DFGLSvf~~~g~~~~~#VGS~YYvAPEVL~+~yGpe~D#WsaGvilYilLsGvPPFWaete~G

CDPKα     QAARLIKTIVEVVEACHSLGVMHRDLKPENFLFDTIDEDAKLKATDFGLSVFYKPGESFCDVVGSPYYVAPEVLRKLYGPESDVWSAGVILYILLSGVPPFWAESEPG
CDPKβ     EAAKLIKTIVGVVEACHSLGVMHRDLKPENFLFDTPGEDAQMKATDFGLSVILQARQAFHDVVGSPYYVAPEVLCKQYGPEVDVWSAGVILYILLSGVPPFWAETEAG
CDPKγ     AAASICRQIVNVVHICHFMGVMHRDLKPENFLLSSRDENALLKATDFGLSVFIEEGKVYRDIVGSAYYVAPEVLRRRCGKEIDIWSAGVILYILLSGVPPFWAETEKG
SPK       KAAELIKIIVGIIETCHSHGVMHRDLKPENFLLLDADDEFSVKAIDFGLSVFFRPGQVFREVVGSPYYIAPEVLEKRYGPEADIWTAGVILYVLLTGVPPFWADTQSG
DsCPK2    AAATICRAVVNVVNICHFMGVMHRDLKPENFLLATKEENAMLKATDFGLSVFIEEGKMYRDIVGSAYYVAPEVLRRNYGKEIDVWSAGVILYILLSGVPPFWAETEKG
DsCPK11   KAAELIRIIVSIVAMCHSLGVMHRDLKPENFLLLDKDDDLSIKAIDFGLSVFFKPGQVFTELVGSPYYVAPEVLHKRYGPESDVWSAGVILYVLLSGVPPFWAETQQG
ZmCDPK    GAAELLRAIVQIVHTCHSMGVMHRDIKPENFLLLSKDEDAPLKATDFGLSVFFKEGELLRDIVGSAYYIAPEVLKRKYGPEADIWSVGVMLYIFLAGVPPFWAENENG
Atcdpk1   AAAAVARTIAEVVMMCHSNGVMHRDLKPENFLFANKKENSPLKAIDFGLSVFFKPGDKFTEIVGSPYYMAPEVLKRDYGPGVDVWSAGVIIYILLCGAPPFWAETEQG
Atcdpk2   EAVKLIKTILGVVEACHSLGVMHRDLKPENFLFDSPKDDAKLKATDFGLSVFYKPGQYLYDVVGSPYYVAPEVLKKCYGPEIDVWSAGVILYILLSGVPPFWAETESG
CPK1      KAAELTRTIVGVVEACHSLGVMHRDLKPENFLFVSKHEDSLLKTIDFGLSMFFKPDDVFTDVVGSPYYVAPEVLRKRYGPEADVWSAGVIVYILLSGVPPFWAETEQG
CPK2      KAAELARTIVGVLEACHSLGVMHRDLKPENFLFVSREEDSLLKTIDFGLSMFFKPDEVFTDVVGSPYYVAPEVLRKRYGPESDVWSAGVIVYILLSGVPPFWAETEQG
CPK3      AAADLCRQMVMVVHSCHSMGVMHRDLKPENFLFLSKDENSPLKATDFGLSVFFKPGDKFKDLVGSAYYVAPEVLKRNYGPEADIWSAGVILYILLSGVPPFWGENETG
CPK4      EAAKLIKTILGVVEACHSLGVMHRDLKPENFLFDSPSDDAKLKATDFGLSVFYKPGQYLYDVVGSPYYVAPEVLKKCYGPEIDVWSAGVILYILLSGVPPFWAETESG
CPK5      KAAELTKIIVGVVEACHSLGVMHRDLKPENFLLVNKDDDPSLKAIDFGLSVFFKPGQIFTDVVGSPYYVAPEVLLKRYGPEADVWTAGVILYILLSGVPPFWAETQQG
CPK6      KAAELTKIIVGVVEACHSLGVMHRDLKPENFLLVNKDDDFSLKAIDFGLSVFFKPGQIFKDVVGSPYYVAPEVLLKHYGPEADVWTAGVILYILLSGVPPFWAETQQG
CPK7      AAAAVMKTIVEVVQICHKQGVMHRDLKPENFLFANKKETSALKAIDFGLSVFFKPGEQFNEIVGSPYYMAPEVLRRNYGPEIDVWSAGVILYILLCGVPPFWAETEQG
CPK8      AAAAVMKTILEVVQICHKHGVMHRDLKPENFLFANKKETSALKAIDFGLSVFFKPGEGFNEIVGSPYYMAPEVLRRNYGPEVDIWSAGVILYILLCGVPPFWAXTEQG
CPK9      AAASVCRQIVNVVKICHFMGVLHRDLKPENFLLSSKDEKALIKATDFGLSVFIEEGKVYRDIVGSAYYVAPEVLRRRYGKEVDIWSAGIILYILLSGVPPFWAETEKG
DcCDPK    AAATICRQIVNVVHVCHFMGVMHRDLKPENFLLSSKDKDAMLKATDFGLSVFIEEGKVYRNIVGSAYYVAPEVLRRSYGKEIDIWSAGVILYILLSGVPPFWAENEKG
```

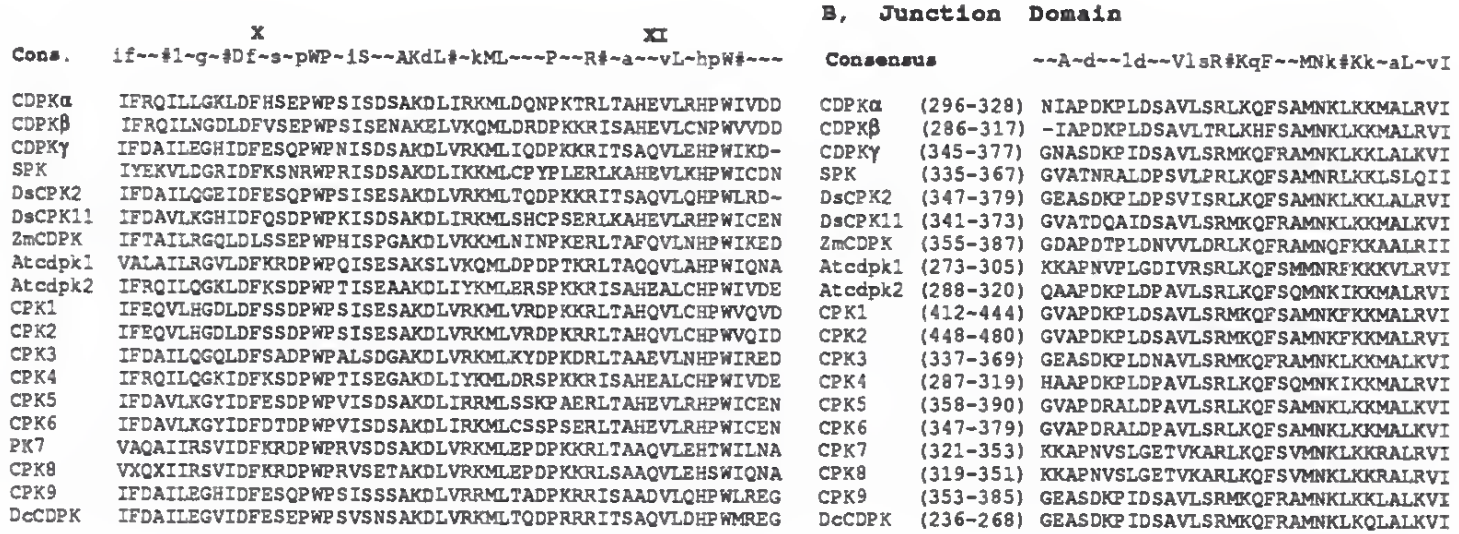

Figure 2-2 continued

C. Calmdoulin-like Domian

```
                        ------I-----                                            -----II-----
Consensus         Ae~lseeEi~glk~#F~-#D-d~-g~it#~ELK~GL~--G~--#----#--lM~AAd~D~-g~idy~EF#~at#h~~k#er~e~l~~AF~~f

CDPKα    (329-508)  AERLSEEEIGGLKELFKMIDTDNSGTITFDELKDGLKRVGS-ELMESEIKDLMDAADIDKSGTIDYGEFIAATVHLNKLEREENLVSAFSYF
CDPKβ    (318-498)  AERLSEEEIGGLKELFKMIDTDNSGTITFEELKEGLKSVGS-NLMESEIKSLMEAADIDNNGSIDYGEFLAATLHLNKMEREENLVAAFAYL
CDPKγ    (378-559)  AENMSAEEIQGLKAMFTNMDTDKSGTITYEELKSGLHRLGS-KLTEAEVKQLMEAADVDGNGSIDYIEFITATMHRHKLERDDQLFKAFQYF
SPK      (368-534)  AERLSEEEIVGLREMFKAMDTKNRSVVTFGELK-GLKRYSS-VFKDTEINDLMEAA-DDTTSTINWEEFIAAAVSLNKIEREKHLMAAFTYF
DsCPK2   (380-533)  ASNLNEEEIKGLKQMFTNMDTDNSGTITYEELKAGLAKLGS-KLSETEIGDIMEAAHNDNNVTIHYEEFIAATLPLNKIEREEHLLAAFTYF
DsCPK11  (374-542)  AERLSEEEIAGLREMFKAVDTKNRGVITFGELREGLRRFGA-EFKDAEVKQLMEAADVDGNGSIDYVEFITATMHRHKLERDEHLFKAFQYF
ZmCDPK   (388-550)  AGCLSEEEITGLKEMFKNIDKDNSGTITLDELKHGLAKHGP-KLSDSEMEKLMEAADADGNGLIDYDEFVTATVHMNKLDREEHLYTAFQYF
Atcdpk1  (306-495)  AEHLSIQEVEVIKNMFSLMDDDKDGKITYPELKAGLQKVGS-QLGEPEIKMLMEVADVDGNGFLDYGEFVAVIIHLQKIENDELFKLAFMFF
Atcdpk2  (321-495)  AERLSEEEIGGLKELFKMIDTDNSGTITFEELKAGLKRVGS-ELMESEIKSLMDAADIDNSGTIDYGEFLAATLHMNKMEREEILVAAFSDF
CPK1     (445-610)  AESLSEEEIAGLKEMFNMIDADKSGQITFEELKAGLKRVGA-NLKESEILDLMQAADVDNSGTIDYKEFIAATLHLNKIEREDHLFAAFTYF
CPK2     (481-646)  AESLSEEEIAGLKQMFKMIDADNSGQITFEELKAGLKRVGA-NLKESEILDLMQAADVDNSGTIDYKEFIAATLHLNKIEREDHLFAAFSYF
CPK3     (370-526)  AENLSEEEIIGLKEMFKSLDTDNNGIVTLEELRTGLPKLGS-KISEAEIRQLMEAADMDGDGSIDYLEFISATMHMNRIEREDHLYTAFQFF
CPK4     (320-501)  AERLSEEEIGGLKELFKMIDTDNSGTITFEELKAGLKRVGS-ELMESEIKSLMDAADIDNSGTIDYGEFLAATLHINKMEREENLVVAFSYF
PK5      (391-556)  AESLSEEEIAGLREMFQAMDTDNSGAITFDELKAGLRKYGS-TLKDTEIHDLMDAADVDNSGTIDYSEFIAATIHLNKLEREEHLVAAFQYF
CPK6     (380-544)  AESLSEEEIAGLRAMFEAMDTDNSGAITFDELKAGLRRYGS-TLKDTEIRDLMEAADVDNSGTIDYSEFIAATIHLNKLEREEHLVSAFQYF
CPK7     (354-535)  AEHLSVEEAAGIKEAFEMMDVNKRGKINLEELKYGLQKAG-QQIADTDLQILMEATDVDGDGTLNYSEFVAVSVHLKKMANDEHLHKAFNFF
CPK8     (352-533)  AEHLSVEEVAGIKEAFEMMDSKKTGKINLEELKFGLHKLGQQQIPDTDLQILMEAADVDGDGTLNYGEFVAVSVHLKKMANDEHLHKAFSFF
CPK9     (386-541)  AENIDTEEIQGLKAMFANIDTDNSGTITYEELKEGLAKLGS-KLTEAEVKQLMDAADVDGNGSIDYIEFITATMHRHRLESNENLYKAFQHF
DcCDPK   (269-425)  AESLSEEEIKGLKSMFANMDTDKSGTITYEELKSGLARLGS-KLSEVEVQQLMDAADVDGNGTIDYLEFITATMHRHKLESYEH--QAFQYF


       -----III----                          ------IV----
Cons.  Dkd~Sg◊It~del--a------D---------- #~~##--#D-dnDGrI-◊~EF-aMM---------------------------------------------

CDPKα    DKDGSGYITLDEIQQACKDFGLDDI--------HIDDMIKEIDQDNDGQIDYGEFAAMM------RKGNGGIGR-RTMRKTLNLR--DALGLVDNGSNQVIEGYFK*
CDPKβ    DKDGSPYITIDELQQACKDFSLGDV--------HLDEMIKEIDQDNDGRIDYAEFAAMM------KKGDPNMGRSRTMKGNLNFNIADAFGMKD-----SS*
CDPKγ    DKDNSGFITRDELESAMKEYGMGDDATIKEIISEVDTIISEVDTDHDGRINYEEFSAMM------KSGN---------QQQGKLF*
SPK      DKDGSGFITVDKLQKACMERNMEDT--------FLEEMILEVDQNNDGQIDYAEFVTMM------QSNNFGLGW-QTVESSLNVALREAPQVY*
DsCPK2   DKDNSGFITRDELESALIEHEMGDTSTIKD-------IISEVDTDNDGRINYEEFCAMM------RGGG--------MQQPMRLK*
DsCPK11  DKDGSGYITVDKLQRACGEHNMEDS--------LLEEIISEVDQNNDGQIDYAEFVAMM------QGSNVGLGW-QTMESSLNVALRDAPQVH*
ZmCDPK   DKDNSGYITKEELEHALKEQGLYDADKIKD-------IISDADSDNDGRIDYSEFVAMM------RKGTAGAEP-MNIKKRRDIVL*
Atcdpk1  DKDGSTYIELDELREA-----LADELGEPDA-SVLSDIMREVDTDKDGRINYDEFVTMMKAGTDWRKASRQYSRERFKSLSINLMKDGSLHLHDALTGQTVPV*
Atcdpk2  DKDGSGYITIDELQSACTEFGLCDT--------PLDDMIKEIDLDNDGKIDFSEFTAMM------RKGD-GVGRSRTMMKNLNFNIADAFGVDG----EKSDD*
CPK1     DKDGSGYITPDELQQACEEFGVEDV--------RIEELMRDVDQDNDGRIDYNEFVAMM------QKGSITGG---PVKMGLEKSFSIALKL*
CPK2     DKDESGFITPDELQQACEEFGVEDA--------RIEEMMRDVDQDKDGRIDYNEFVAMM------QKGSIMGG---PVKMGLENSISISLKH*
CPK3     DNDNSGYITMEELELAMKKYNMGDDKSIKE-------IIAEVDTDRDGKINYEEFVAMM------KKG----NP-ELVPNRRRM*
CPK4     DKDGSGYITIDELQQACTEFGLCDT--------PLDDMIKEIDLDNDGKIDFSEFTAMM------KKGD-GVGRSRTMRNNLNFNIAEAFGVEDTSSTAKSDDSPK*
CPK5     DKDGSGFITIDELQQACVEHGMADV--------FLEDIIKEVDQNNDGKIDYGEFVEMM------QKGNAGVGR-RTMRNSLNISMRDA*
CPK6     DKDGSGYITIDELQQSCIEHGMTDV--------FLEDIIKEVDQDNDGRIDYEEFVAMM------QKGNAGVGR-RTMKNSLNISMRDV*
CPK7     DQNQSGYIEIDELREA-----LNDELDNTSSEEVIAAIMQDVDTDKDGRISYEEFVAMMKAGTDWRKASRQYSRERFNSLSLKLMRDGSLQLEGET*
CPK8     DQNQSDYIEIEELREA-----LNDEVD-TNSEEVVAAIMQDVDTDKDGRISYEEFAAMMKAGTDWRKASRQYSRERFNSLSLKLMREGSLQLEGEN*
CPK9     DKDSSGYITIDELESALKEYGMGDDATIKE-------VLSDVDSDNDGRINYEEFCAMM------RSGNP-------QQQQPRLF*
DcCDPK   DKDNSGFITKDELESAMKEYGMGDEATIKD-------IISEVDSDNDGRINYDEFCAMM------RRAR---------NRR-KLFSVSLTS*
```

Figure 2-2 continued

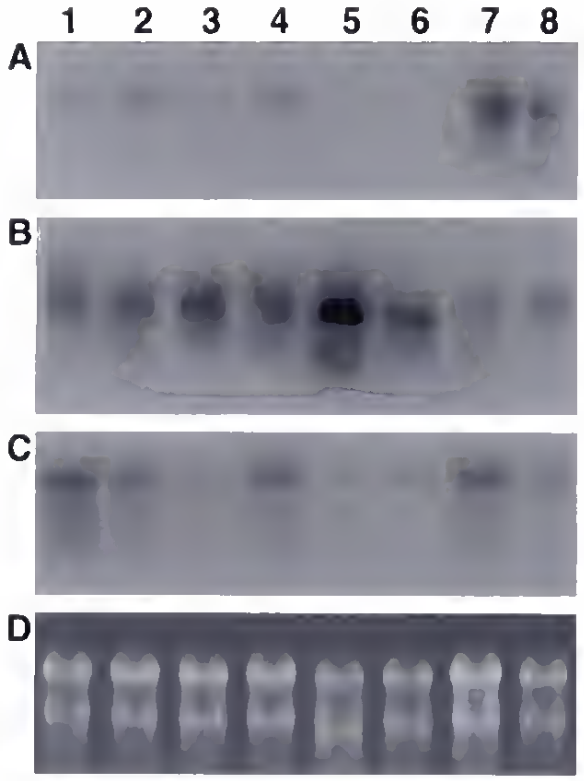

Figure 2-4. RNA blot hybridization analyses of CDPK encoding genes. Total RNA (10 μg) from three-week-old soybean plants (lanes 1-5), eight-day-old seedlings, (lane 6), and soybean cell cultures (lanes 7-8), was fractionated in a formaldehyde agarose gel, blotted, and hybridized with specific probes for each enzyme as described in Experimental Procedures. The samples were : lane 1, root; lane 2, stem; lane 3, shoot tip; lane 4, petiole; lane 5, leaf; lane 6, whole seedling; lane 7, three-day-old cell culture; and lane 8, seven-day-old cell culture.
a) CDPKα ; b) CDPKβ; c) CDPKγ; d) Ethidium bromide stained gel demonstrates that the equal amounts of RNA were loaded in each lane.

week-old plants, in eight-day-old seedlings, and in suspension cell cultures of different ages. However, the relative distribution of each transcript differed. CDPKα mRNA was most highly expressed in three-day-old cell culture. This result may imply that the expression of CDPKα is regulated by developmental stages. CDPKβ which was originally isolated from a plumule library, was most highly expressed in leaves. CDPKγ, which was originally isolated from a library made from tissues enriched in nitrogen-fixing root nodules, was most highly expressed in roots. It will be interesting to examine if the transcription levels of each isoform in different tissues correlates with the translation levels in order to better understand what physiological implications can be made from these observations.

Expression of CDPK Fusion Proteins in *E. coli* and Purification

As a step towards determining if the three soybean CDPKs perform distinct or overlapping roles in the cell, the kinetic properties of recombinant enzymes expressed in *E. coli* were examined. The three full length enzymes and an amino terminal deletion mutant of CDPKγ, CDPK$\gamma_{(66\text{-}538)}$, were expressed as glutathione *S*-transferase (GST) fusion proteins. These recombinant proteins were highly expressed in *E. coli* under experimental conditions and showed CDPK activity when total cell extracts were used for enzyme assays (data not shown). Interestingly, it was found that monoclonal antibodies crossreact with CDPKβ very weakly (Figure 2-4). Conversely, polyclonal antibodies raised against the CLD domain of CDPKα (Bachmann et al., 1996) recognized both CDPKβ and γ quite well.

Recombinant enzymes were highly purified by affinity chromatography on glutathione-agarose and anion exchange chromatography (Figure 2-5). The activities of CDPKα, and β were stable in the absence of

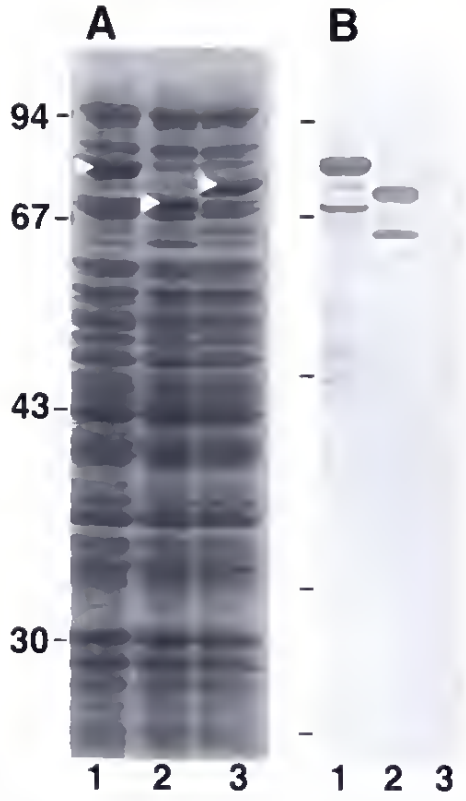

Figure 2-4. Immunoblot of recombinant proteins with monoclonal antibodies. Lane 1 contains GST-CDPKg; lane 2, GST-CDPK$_{g(66-538)}$; lane 3, GST-CDPKb.
a) Cells expressing recombinant proteins were resolved in 10 % SDS-PAGE and stained with Coomassie blue or b) blotted to nitrocellulose and probed with monoclonal antibodies directed against native soybean cell CDPK.

Figure 2-5. SDS-PAGE of purified recombinant proteins. Purified recombinant proteins (5 μg) were analyzed by electrophoresis in 10 % SDS-polyacrylamide gel and stained with Coomassie blue. Lane 1 contains GST-CDPKγ; lane 2, GST-CDPKγ$_{(66-538)}$; lane 3, GST-CDPKβ. Molecular mass markers are as follows (in kilodaltons): phosphorylase b, 94; BSA, 67; ovalbumin, 43; carbonic anhydrase, 30.

reducing agents and at low ionic strength, and these enzymes could be stored in 50% (v/v) glycerol at -80 °C without appreciable loss of activity. CDPKγ and CDPK$\gamma_{(66\text{-}538)}$, on the other hand, required reducing agents and 300 mM KCl or NaCl during dialysis and could not be frozen without almost complete loss of activity. These enzymes were also expressed as His_6-fusion proteins, but because His_6-CDPKγ required high ionic strength to maintain solubility and activity, further purification of this protein by anion-exchange chromatography was not possible. In addition, His_6-CDPK$\gamma_{(66\text{-}538)}$ bound to phenyl-Sepharose at low ionic strength in the presence of Ca^{2+}, but could be eluted only by 6 M urea.

Substrate Specificities and Kinetic Parameters

The activities of soybean CDPKs with various synthetic peptides and histone IIIS were determined in the presence or absence of Ca^{2+} (Table 2-1). There was little difference in the activities of CDPKγ and the amino terminal deletion mutant CDPK$\gamma_{(66\text{-}538)}$, showing that the N-terminal 66 residues of this isoenzyme are not required for catalytic activity.

Histone IIIS, a good substrate for CDPK purified from soybean cell culture (Putnam-Evans et al., 1990), was phosphorylated by all three isozymes (Table 2-1). Other proteins such as casein and bovine serum albumin were not phosphorylated by any of the recombinant isoenzymes (data not shown).

Substrate peptides containing the motif basic-X-X-Ser/Thr, in which the basic residue is arginine or lysine, x is any residue, and serine or threonine is the phosphorylated residue, are good substrates for CDPKs (Roberts and Harmon, 1992). The specific activities of the soybean CDPKs with 100 μM of each of four peptides containing this motif, syntide-2, autocamtide-2, and substrate peptides of skeletal and smooth muscle myosin

Table 2-1. Activity of CDPK isoforms with various substrates.

Enzyme activitites were measured as described in Experimental Procedures with 0.5 mg/ml histone IIIS or 100 μM synthetic peptide in the presence of 1 mM EGTA (-Ca^{2+}) or 1 mM EGTA plus 1.1 mM Ca^{2+} (+ Ca^{2+})[a].

enzyme	Ca^{2+}	Histone IIIS	Syntide-2	Autocamtide	MLCKsk	MLCKsm
				μmol/min/mg		
CDPKα	+	0.045	1.67	0.81	0.15	0.14
	–	nd[b]	0.075	0.025	0.005	0.005
CDPKβ	+	0.45	3.55	2.41	0.98	1.82
	–	0.02	0.14	0.07	0.03	0.08
CDPKγ	+	0.25	1.86	1.82	1.92	1.57
	–	nd[b]	0.03	0.01	0.004	0.03
$\gamma_{(66\text{-}538)}$	+	0.28	1.89	2.12	1.65	1.56
	–	nd[b]	0.02	0.008	0.007	0.008

[a]The standard error of each mean value of enzyme activities was less than 10% of the tabulated mean value.
[b]nd, not detectable.

light chain kinases, were compared (Table 2-1). Phosphorylation of the peptide substrates by CDPKγ and CDPKγ(66-538) was stimulated 52 to 480-fold, whereas the activities of CDPKα and CDPKβ with these substrates was stimulated 22- to 30-fold. All four peptides were good substrates for CDPKγ and CDPKγ(66-538), and the maximal activity of these enzymes with each peptide varied little (<1.4-fold). The activities of CDPKα and CDPKβ varied by as much as 12- and 3.6-fold, respectively. Syntide-2 and autocamtide were good substrates for both CDPKα and CDPKβ. In contrast, MLCK substrate peptides were good substrates for CDPKβ, but not for CDPKα.

To examine the basis for the difference in substrate preference, the kinetic parameters for the CDPKs with two of the peptide substrates were determined (Table 2-2). The apparent K_m and V_{max} of CDPKα and CDPKγ with syntide-2 as substrate were similar and about 2-fold lower than the parameters for CDPKβ. Comparison of the ratio of V_{max} to K_m, which is a measure of catalytic efficiency, shows that syntide-2 is an equally good substrate for all three isoenzymes. In contrast, skeletal muscle myosin light chain kinase peptide is a good substrate for only CDPKγ. The kinetic parameters for the two peptides with CDPKγ are similar and there is only a two-fold difference in the catalytic efficiencies. While the apparent V_{max}s for CDPKα and CDPKβ were 3- and 2-fold higher, respectively, than that of CDPKγ, the apparent K_ms were 140- and 10-fold higher, respectively. Comparison of the ratios of V_{max} to K_m, shows that CDPKα has a strong preference for syntide-2, CDPKβ has less difference in preference, and CDPKγ has a slight preference for syntide-2. Syntide-2 and autocamtide-2 contain branched-chain amino acid residues at positions P-5, P+1, and P+4, whereas the MLCK peptides do not (Figure 2-6). One or more of these residues may be determinants for phosphorylation of a protei by CDPKα.

Table 2-2. Kinetic parameters of CDPK isoforms.

Values of apparent Vmax and K_m were determined from double-reciprocal plots.

	Syntide-2			MLCKsk		
Enzyme	Vmax	K_m	$\frac{Vmax}{K_m}$	Vmax	K_m	$\frac{Vmax}{K_m}$
	μmol/min/mg	*μM*		*μmol/min/mg*	*μM*	
CDPKα	2.4 ± 0.3	18.2 ± 1.5	0.13	5.7 ± 2.5	3700 ± 1900	0.001
CDPKβ	5.5 ± 0.7	34.2 ± 1.0	0.16	3.8 ± 0.8	282 ± 46	0.013
CDPKγ	2.5 ± 0.1	16.6 ± 0.7	0.15	2.0 ± 0.1	27 ± 0.8	0.074

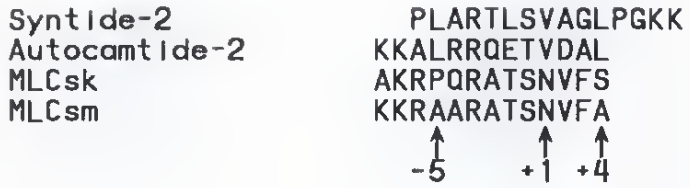

Figure 2-6. Sequences of Substrate Peptides. Residues are numbered relative to the phosphorylated residue at position P. Residues amino terminal to P have negative numbers and those carboxyl terminal to P have positive numbers.

Effect of pH

The effect of pH on phosphorylation of syntide 2 by the three CDPKs is shown in Figure 2-7. All three isoenzymes showed maximal activity at pH 7 to 8, but CDPKα and β were more tolerant to alkaline conditions. The broad pH optimum of CDPKα and β is similar to that of CDPK purified from soybean (Putnam-Evans et al., 1990).

Effect of Protein Kinase Inhibitors

The effect of several classes of protein kinase inhibitors on the activity of soybean CDPKs was determined. Control assays containing 0.01% (v/v) DMSO did not affect kinase activity. Staurosporine, an inhibitor of broad specificity that is suggested to interact with an essential region of catalytic domain of protein kinases (Tamaoki, 1991), inhibited the CDPKs (Table 2-3) with IC_{50}s between 70 and 120 nM. Another general inhibitor of protein kinases is K-252a (Kase et al., 1987), which competes with ATP. K-252a inhibited the CDPKs (Table 2-3), with IC50s between 300 and 800 nM. The IC_{50}s for both of these inhibitors were one to two orders of magnitude higher than those observed with PKC, PKA, or MLCK (Hashimoto et al., 1991; Kase et al., 1987; Tamaoki, 1991).

Table 2-3. IC_{50} Values for Inhibitors of CDPK Isoenzymes.

Inhibitors	CDPKα	CDPKβ	CDPKγ
		(μM)	
Staurosporine	0.11	0.12	0.07
K-252a	0.8	0.8	0.3
Calphostin C	9.0	5.0	1.6

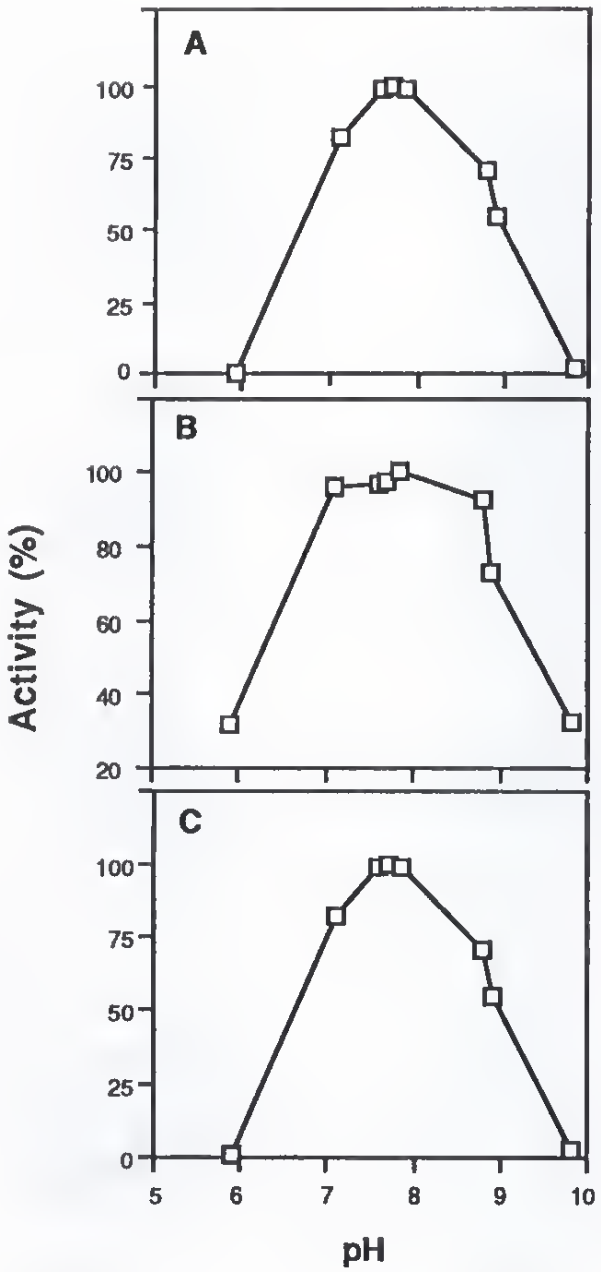

Figure 2-7. Effect of pH on activity. Enzymes were assayed with syntide-2 as described in Experimental Procedures. Buffers (50 mM) were: MES (pH 5.9), HEPES (pH 7.1, 7.9), Tris (pH 7.6, 7.7, 8.8), and CHES (pH 8.9, 9.8). a) CDPKα ; b) CDPKβ; c) CDPKγ.

Highly effective inhibitors of CaMKII and myosin light chain kinase had little to no effect on the CDPKs; concentrations of these inhibitors used in the assays were 50 μM H8 (Hidaka et al., 1984), 10 μM KN62 (Hidaka et al., 1991), 30 μM KN93 (Sumi et al., 1991), and 50 μM ML7 (Saitoh et al., 1987). Calphostin C, which is reported to specifically inhibit protein kinase C through interaction with its regulatory domain, inhibited all three CDPK isoforms (Table 2-3), but the IC_{50} values were one to two orders of magnitude greater than those for protein kinase C (Tamaoki, 1991).

Purification of Anti-Ntγ Antibodies

First, purification of polyclonal antibodies (Anti-Ntγ) raised against N-terminal domain of CDPKγ fused to GST were attempted from immunoblots. (Olmsted, 1981; Smith and Fisher, 1984). But Anti-Ntγ could not be eluted effectively due to the high affinity (data not shown). Therefore, an affinity column was prepared by coupling GST expressed in *E. coli* to AminoLink™ Gel (Pierce) in order to absorb anti-GST antibodies. Partially purified Anti-Ntγ did not crossreact with GST nor with recombinant CDPKα nor β, but did crossreact with many protein bands in soybean cell extracts. When His_6 fusion proteins of full-length and an N-terminal deletion mutant of CDPKγ were used as antigens on blots to elute antibodies, antibodies recognizing N-terminal mutant were eluted. This result suggested that antibodies recognizing amino acid residues from 66 to 83 of the N-terminal domain of CDPKγ could be affinity purified. Therefore, Anti-Ntγ were purified further using affinity column made from highly purified His_6-$CDPK\gamma_{(66\text{-}538)}$. When soybean cell extracts were immunostained with affinity purified Anti-Ntγ, nonspecific cross reactions were reduced, and very faint band of right size to be CDPKγ could be detectable (data not shown).

To test whether CDPKγ is localized at membranes, microsome and cytosol fractions were challenged with Anti-Ntγ and anti-CLD antibodies. The blots (Figure 2-8) suggested that CDPKγ is probably located both in microsome and cytosol and probably at low abundance.

Discussion

Three Soybean CDPKs are Constitutively Expressed Multifunctional Protein Kinases

The primary structures of soybean CDPKα, β, and γ are similar to those of CDPKs from a variety of plants. The most distinctive feature of the new CDPKs is the eight amino acid insert prior to the fourth EF-hand that is present in CDPKγ, but not CDPKα and β. Whether this feature contributes to the functional properties of this CDPKγ remains to be determined. In contrast to a CDPK from maize that is expressed only in pollen (Estruch et al., 1994), the CDPKs examined in this study are present in numerous parts of soybean plant. These CDPKs are also expressed in cell cultures and young plants grown under standard conditions. Expression of a CDPK from *Arabidopsis* is induced by environmental stress (Urao et al., 1994), and another from mung bean is induced by mechanical strain or auxin (Botella et al., 1996).

We have shown that biochemical properties and substrate specificities of soybean CDPK isoenzymes differ. Biochemical properties of CDPKα and β are more similar to each other than to those of CDPKγ as the sequence identity between CDPKα and β is higher than that between CDPKα and γ or β and γ. In contrast to CDPKα and CDPKβ, CDPKγ is not stable in the absence of DTT, in buffers of low ionic strength, or when frozen. All three isoenzymes phosphorylate peptides containing a basic-X-X-Ser motif, but each isoenzyme

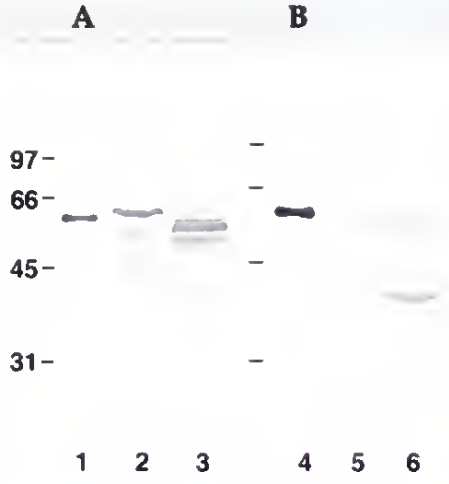

Figure 2-8. Immunoblots of soybean cell extracts with Anti-Ntγ or anti-CLD antibodies. Lane 1 contains 20 ng of purified His_6-CDPKγ; lane 2, 25 μg of microsomal fraction prepared from soybean cells, and lane 3, 25 μg of soluble fraction.
a) Cell extracts were resolved in 10 % SDS-PAGE and blotted onto nitrocellulose membrane and probed with anti-CLD antibodies or b) with Anti-Ntγ.

has a different value of apparent K_m and V_{max} for each of the peptides. CDPKα selectively phosphorylates syntide-2, and CDPKγ phosphorylates the four peptides tested equally well.

Recently, Bachmann et al., (Bachmann et al., 1996) showed that serine-543 in nitrate reductase is phosphorylated by partially purified CDPK from spinach. Analysis of synthetic peptides in which residues surrounding the phosphorylation site were varied, showed that motif preferred by the spinach CDPK was hydrophobic-X-basic-X-X-Ser, where the hydrophobic residue at P-5 was leucine. These observations agree with the conclusion that the presence of branched chain aliphatic amino acids at one or more of the positions P-5, P+1, and P+4 is determinant for phosphorylation by some CDPK isoenzymes. However, a systematic analysis of phosphorylation motifs is needed in order to define the importance of the hydrophobic residues for each isoenzyme.

None of the recombinant CDPKs is identical to CDPK purified from soybean cell culture. Unlike the recombinant CDPKs, the native enzyme phosphorylates histone IIIS very well (Harmon et al., 1996), but its peptide substrate specificity is most similar to that of CDPKα. The amino acid sequences of peptides derived from the native enzyme (Harper et al., 1991) are not identical to sequences of CDPKα, β, or γ, but 60 of 62 of the known residues match the sequence of mung bean CDPK (Botella et al., 1996). While it is possible that the properties of the recombinant enzymes differ from the native soybean enzymes because of modifications due to their expression in a prokaryotic system, the high identity of the known sequence from native enzyme with the mung bean CDPK suggest that the native soybean cell enzyme is a fourth isoenzyme that was purified to near homogeneity because of its distinct biochemical properties.

Subcellular Localization of CDPKs Remains Unsolved

While there is evidence for localization of CDPKs with microfilaments in onion and spiderwort (Putnam-Evans et al., 1989) and with plasma membranes of soybean (Borochov-Neori and Harmon, 1993), oat (Schaller et al., 1992), and zucchini (Verhey et al., 1993), the basis for these associations is not known. None of the CDPKs characterized to date contain membrane spanning domains. Some CDPKs such as CPK1 (Harper et al., 1993) and CPK2 (Hrabak et al., 1996) from *Arabidopsis* and three rice CDPKs (Breviario et al., 1995; Kawasaki et al., 1993) have putative myristoylation sites, and the myristoylation of recombinant carrot CDPK has been reported (Farmer and Choi, 1995), but it is not yet known if myristoylation is sufficient for membrane localization. The localization of protein kinases to specific sites in cells via interaction of the enzyme with targeting proteins is a common theme in signal transduction in animals (Mochly-Rosen, 1995).

The amino terminal domain of CDPKγ is a candidate for such interaction since it is relatively long, and its deletion does not affect the catalytic activity of the enzyme. If isoform specific antibodies can be obtaind they will be valuable tools to elucidate the subcellular localization of CDPK isoforms. In this regard, the production and purification of antibodies specific to CDPKγ was attempted. Raising polyclonal antibody against GST tagged N-terminal domain of CDPKγ that was expressed in *E. coli.* was successful. The polyclonal antibody only recognized recombinant CDPKγ, but not α or β. However, affinity purification of the antibody by conventional methods was difficult due to its high affinity. These antibodies were shown to crossreact with a band of the right size for CDPKγ, but also recognized other bands in crude extracts in either cytosolic or microsomal fractions of soybean cells.

Proving that the right sized band recognized by the antibodies corresponds to CDPKγ will not be an easy task because of extreme instability of the enzyme.

General Protein Kinase Inhibitors Shows Higher IC_{50}s for CDPKs than Those for Animal Protein Kinases

CDPKα, β, and γ differed in their susceptibility to inhibition by protein kinase inhibitors, e. g., staurosporine, K-252a, and calphostin C. CDPKγ was the most sensitive to inhibition by all three compounds. The IC_{50}s for staurosporine and K-252a were higher than those required for inhibition of the animal protein kinases, PKA, PKC, MLCK, or CaMKII, but these concentrations are similar to those needed for inhibition of various responses of plant cells to stimuli (Hashimoto et al., 1991; Kase et al., 1987; Tamaoki, 1991). Calphostin C also inhibited the soybean CDPKs, but with IC_{50}s 30- to 200-fold higher than that for PKC. These results show that the concentration of calphostin C used in experiments with plant cells must be carefully considered before it can be concluded that an observed effect resulted from inhibition of a plant protein kinase C homolog. KN62 inhibits CaMKII competitively with respect to calmodulin; however, it did not show any significant inhibition of CDPKs at a concentration (10 μM) that inhibits over 80% of CaMKII activity. It will be beneficial to find CDPK-specific inhibitors, either from pharmacological screening or from designing peptides, in order to facilitate studies unraveling the physiological roles of CDPK *in vivo*.

The results demonstrated that three soybean CDPK isoforms differ in biochemical and kinetic properties and in their RNA expression patterns. Although all three CDPKs phosphorylate peptides having the same core motif; each isoform differs in its selectivity for residues surrounding this motif, basal activity, and fold-activation. These results support the hypothesis

that CDPK isoenzymes play distinct or overlapping roles and are not redundant. Another factor that may contribute to distinct roles of CDPK isoforms in transmitting Ca^{2+} signals generated by different stimuli would be the targeting of CDPKs to specific sites differing calcium-sensitivity. Characterization of these latter properties is described in following chapter.

CHAPTER 3
CALCIUM BINDING PROPERTIES OF THREE SOYBEAN CDPKS

Introduction

One of the best studied calcium binding proteins is calmodulin. Calmodulin is an ubiquitous protein in eukaryotes that belongs to the super family of EF-hand proteins (Kretsinger, 1987; Moncrief, 1990; Nakayama, 1994; Kretsinger, 1996) and it is involved in numerous cellular processes that are regulated by Ca^{2+} (Cheung, 1980).

The crystal structure of the Ca^{2+}-calmodulin complex demonstrated that it attains a dumbbell shape: at each end of the dumbbell is a pair of EF-hands and they are connected by a long α helix (Babu et al., 1988; Babu et al., 1985). Comparison of X-ray solution scattering data of calmodulin and troponin C (Heidorn and Trewhella, 1988) and studies from site-directed mutagenesis of the central helix of calmodulin (Persechini and Kretsinger, 1988) predicted that the central helix of calmodulin may form a flexible tether. NMR spectroscopic studies provided the first solution structure of a ternary complex, i.e., Ca^{2+}-CaM and its target peptide (calmodulin binding domain of skeletal myosin light chain kinase) (Ikura et al., 1992). Two crystal structures of the ternary complex between Ca^{2+}-CaM and a smooth muscle myosin light chain kinase peptide (Meador et al., 1992) or a CaM-dependent protein kinase II peptide (Meador et al., 1993) followed. These studies showed that the central helix was indeed bent. The conformational flexibility of the central helix brings the two globular domains closer to each other in order to interact

with a target peptide. These findings confirmed that calmodulin can accommodate specific binding to a large number of targets with high affinity through hydrophobic residues in the two lobes. These hydrophobic pockets are exposed by conformational changes upon binding Ca^{2+} and form a tunnel which wraps around the target peptide due to the flexibility of the central helix (Finn and Forsen, 1995; James et al., 1995; Torok and Whitaker, 1994). However, the binding of Ca^{2+}/calmodulin to its numerous target enzymes with high affinity is rather unusual because the binding sites do not show any sequence conservation.

The Ca^{2+}-binding properties of calmodulin have been the subject of numerous studies for decades since the first report by Teo & Wang (1973). The determination of Ca^{2+}-dissociation constants of calmodulin were reported using equilibrium dialysis (Cox et al., 1981; Crouch and Klee, 1980; Potter et al., 1983), or flow dialysis (Haiech et al., 1981; Haiech et al., 1980; Starovasnik et al., 1993), or a titration technique with a chromophoric Ca^{2+} chelator (Linse et al., 1991). Whether there is a cooperativity among Ca^{2+}-binding sites has been a subject of controversy, however it is now generally agreed that there is positive cooperativity between the two EF-hands in each pair (N-terminal and C-terminal domains of calmodulin) and that the C-terminal domain has about 6-8 fold higher Ca^{2+}-binding affinity than the N-terminal domain (Linse et al., 1991; Porumb, 1994). The largest conformational change occurs upon binding of two Ca^{2+} to the C-terminal domain (Crouch and Klee, 1980). Notably each N-terminal or C-terminal domain maintains its Ca^{2+}-binding property when the domains are separated by tryptic digestion (Linse et al., 1991). This result implies that each domain binds Ca^{2+} independently, although a study employing a series of site-directed mutageneses of the conserved EF-hands indicated that mutations in N-

terminal domain can affect the conformational change in C-terminal domain (Beckingham, 1991; Maune, 1992). The apparent Ca^{2+}-dissociation constants (Kd) for the high-affinity (C-terminal) and the low-affinity (N-terminal) domains are 1 μM and 10 μM, respectively (Linse et al., 1991; Porumb, 1994). However, it is note worthy that the interaction between the two domains of calmodulin is required for the high affinity Ca^{2+}-binding in the presence of a target peptide (Yazawa et al., 1992).

The C-terminal domain of CDPK contains four EF-hands similar to calmodulin (Harper et al., 1991). The activity of CDPK is regulated by Ca^{2+} through this C-terminal domain which was named calmodulin-like domain (CLD) (Yoo et al., 1996). The Ca^{2+}-binding properties and the effects of mutating each EF-hand of PfCDPK from *Plasmodium falcifarum* have been reported (Zhao et al., 1994). Equilibrium dialysis showed that PfCDPK is able to bind four Ca^{2+} per molecule with mean Kd of 80 μM. The $K_{0.5}$ for Ca^{2+} was 15 μM. The binding studies suggested at this concentration of free Ca^{2+} 1 mole of Ca^{2+} binds per mole of PfCDPK. The highly conserved glutamate residue at position 12 in the first and second EF-hands of PfCDPK was crucial for the structural change and for the enzyme activity. Similar studies employing point mutations with *Drosophila* calmodulin revealed that the mutations to the second and fourth Ca^{2+}-binding sites resulted in more deleterious effect than the mutations to the first and third Ca^{2+}-binding sites (Maune, 1992). However, the mutated calmodulin with altered Ca^{2+}-binding properties was able to activate substrate enzymes (Haiech et al., 1991). Yeast calmodulin is similar to vertebrate calmodulin (60% amino acid sequence identity) but only three EF-hands are functional (Starovasniket al., 1993). Intriguingly, mutant yeast calmodulins in binding Ca^{2+} supported the growth of yeast while deleting the calmodulin gene was fatal (Geiser et al., 1991).

Although yeast calmodulin is required for growth, it can perform its function without the apparent ability to bind Ca^{2+}.

CDPK is able to sense directly a Ca^{2+} signal through its calmodulin-like domain. In addition, it is a kinase, an important signaling component, that can act to amplify the signal. Since CDPKs are encoded by a large gene family (Estelle, et al., 1996), one can hypothesize that Ca^{2+} can bring about certain physiological responses in plants by utilizing different CDPK isoforms as the signal mediator(s). CDPK isoforms with different Ca^{2+} sensitivities to decode different Ca^{2+} signals.

There are large number of reports regarding the involvement of Ca^{2+} in various physiological responses of plants (Bush et al., 1996). However, studies demonstrating the roles of CDPKs in plants are limited. Studies on the differences in Ca^{2+} sensitivity of CDPK isoforms, therefore, will be essential to give insight into how different CDPK isoforms may play roles in transducing Ca^{2+} signals. In the present study the direct measurement of Ca^{2+}-binding properties by the flow dialysis method of three soybean CDPKs were undertaken. The flow dialysis method was chosen because it produces a complete data set from a single protein sample in a short time. The rate of Ca^{2+} flux across the dialysis membrane to reach a steady state is within a couple of minutes (Colowick and Womack, 1969; Porumb, 1994; Womack and Colowick, 1973). In addition, this method has been used in numerous studies of calmodulin (Haiech, et al., 1981, 1991; Starovasnik, et al., 1993;Yazawa, et al., 1992). The effect of Ca^{2+} concentrations on the substrate phosphorylation and autophosphorylation were also determined. The concentration of free Ca^{2+} in the activity assays was set by Ca^{2+} buffers.

Experimental Procedures

Materials

Analytical standards of 100 mM $CaCl_2$ and 1 M $MgCl_2$ were purchased from Orion and Fluka, respectively. Solutions of $^{45}CaCl_2$ (29.6 Ci/g, 10 mCi/mL) and [γ-^{32}P]ATP were supplied by DuPont NEN. Spectra/Por® Macrodialyzer and dialysis tubings (molecular cutoff ~3,500) for flow dialysis were obtained from Spectrum Co. Chelex 100 was purchased from Bio-Rad. Fluo-3 pentapotassium salt was from Molecular Probes. All other chemicals were reagent grade or higher.

Protein Purification

Molecular cloning of plasmid constructs for the expression of GST-fusion proteins of soybean CDPK isozymes were described in Chapter 2. Bacterial expressions of recombinant proteins were scaled up to several liters to obtain enough protein for the flow dialysis experiments. The induction conditions were optimized to get proteins in high yield: IPTG was added to 0.5 mM when OD_{600} reached ~0.5 and cells were incubated for 3 hours of induction at low temperature (~20 °C). Depending on the construct, ~0.5-6 mg of proteins/liter of bacterial culture could be obtained. Protein purification procedures were the same as described in Chapter 1 except that the equilibration buffer for ionic exchange chromatography contained only 20 mM Tris, pH 8.0.

Removal of Contaminating Calcium

Glass containers were avoided in the experiments involving calcium binding measurements. Plastic bottles were filled with deionized water and autoclaved. Small containers and graduated cylinders were soaked in 0.1 M HCl and rinsed thoroughly with deionized water. Water and solutions used for flow dialysis were decalcified by passing through a Chelex-100 column (Crouch and Klee, 1980). The membranes for the flow dialysis were stored in 10 mM EGTA at 4 °C and thoroughly rinsed with deionized water followed by final washes in Chelex-treated water prior to flow dialysis. Stock solutions of enzymes were dialyzed in Chelex-treated buffer A (50 mM HEPES, pH 7.5, 100 mM KCl) in the presence or absence of 14.4 mM mercaptoethanol at 4 °C overnight and passed through Chelex column equilibrated with buffer A (Stemmer and Klee, 1994). Following the Chelex-treatment the protein samples were concentrated using a centrifugal concentrator (Centricon-10, Amicon) which was prewashed with Chelex-treated buffer A.

Calcium Measurements

Contaminating Ca^{2+} in water, buffers, and protein samples was measured using fluorescent Ca^{2+} indicator Fluo-3 (Minta et al., 1989), as described elsewhere (Eberhard and Erne, 1991 and 1994). Calcium calibration buffers from Molecular Probes were used for generating a standard curve following the recommendations of the supplier. Briefly, Fluo-3 was dissolved in DMSO to make 1 mM stock. Aliquots of 200 µL each in microcentrifuge tubes were stored at -80 °C. Fluo-3 (0.5-5 µM) was added to 2 ml sample for the measurement of free calcium. A fluorometer cuvette was soaked in 0.1 M HCl for at least 30 minutes and rinsed thoroughly with deionized water

before the fluorescence measurement. Free calcium concentrations in Ca^{2+}/EGTA buffers containing <1 μM free calcium were verified also by using Fluo-3. The contaminating calcium concentration in Chelex-treated flow dialysis buffer (50 mM HEPES and 100 mM KCl) and protein solutions was less than 0.3 μM according to the fluorescence measurement with Fluo-3 and atomic absorption spectrometry. Fluorescence was measured with Perkin-Elmer LS5 spectrofluorometer. Concentrations of various $CaCl_2$ stock solutions that were made by diluting 100 mM $CaCl_2$ in Chelex-treated water and kinase assay buffers containing > 1 μM free calcium were confirmed by atomic absorption spectroscopy.

Calcium Binding Studies

The calcium binding properties of CDPK isoenzymes were studied using flow dialysis method (Colowick and Womack, 1969; Womack and Colowick, 1973; Porumb, 1994). The experiments were performed using a dialysis apparatus (Spectra/Por® Macrodialyzer) at room temperature (24 ± 2 °C). The apparatus consisted of two dialysis cells that were separated by a dialysis membrane. The upper cell chamber contained 7 μM to 30 μM of metal-free protein sample in 1 ml buffer A (50 mM HEPES, pH 7.5, 100 mM KCl) and the lower chamber was filled with 1 ml of buffer A and continuously pumped to the effluent collector (Figure 3-1).

The additions of solutions into the upper chamber were made through a port using ultra-thin gel loading pipette tips (United Scientific Products). The solutions were constantly mixed with a magnetic stirring bar (7 mm X 2 mm) in each chamber. The buffer was pumped from the lower chamber using the multistaltic pump (Buchler Instruments) at a flow rate of 3 ml/min. The Ca^{2+} titration was initiated by adding an aliquot of 1.5 μM to 7.5 μM

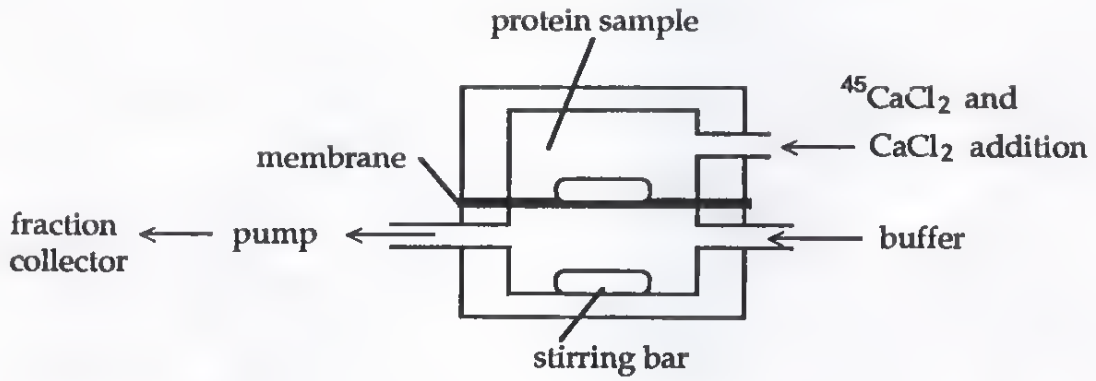

Figure 3-1. A schematic representation of flow dialysis system.
The flow dialysis apparatus (Spectra/Por®MacroDialyzer) was supplied from Spectrum. The upper and lower cell chambers each can hold 1 ml of sample. The upper cell contained protein sample in dialysis buffer. Injection of Ca^{2+} was made through an open port as indicated. The lower cell contained dialysis buffer which was continuously pumped from a buffer reservoir to the fraction collector by multistaltic pump as explained in the Experimental procedures.

$^{45}CaCl_2$ to the protein sample in the upper chamber and continued by adding 1.25 µl to 4 µl of different $CaCl_2$ stock solutions in each cycle. The time lag from the moment when the buffer left the lower chamber until it reached the fraction collector was measured and the correct timing of $CaCl_2$ addition was adjusted accordingly. The final chase-out was carried out with 4 mM $CaCl_2$. The effluent was collected in 1.5 ml fractions in microcentrifuge tubes every 30 seconds. Each was mixed with 2 ml of SintiVerse (Fisher) for the radioactivity measurement in the Scintilation counter (Beckman). Steady states of radioactivity were reached at the third fraction of each cycle. The average counts from the last two fractions (3rd and 4th) of each cycle were taken to calculate free calcium concentration from known initial calcium concentration. The moles of bound calcium per mole of protein was calculated by combining the concentration of bound calcium and protein concentration as described by Porumb, 1994, i.e.,

$$[CaCl_2]_i^{free} = [CaCl_2]_i^{total} \times \frac{(volume)_{initial}}{(volume)_i} \times \frac{\{cpm\}_i}{\{cpm\}_F}$$

$$[CaCl_2]_i^{bound} = [CaCl_2]_i^{total} - [CaCl_2]_i^{free}$$

$$\frac{(\text{mole of bound } CaCl_2)_i}{\text{mole of protein}} = \frac{[CaCl_2]_i^{bound}}{[\text{protein}]}$$

where i and F represent i th cycle and final chase-out, respectively. It was assumed that there is no loss of $^{45}CaCl_2$ by diffusion through the membrane and thus the volume change in each cycle by the calcium addition was substituted for $^{45}CaCl_2$ concentration change in the calculation. Control experiments in the absence of protein sample confirmed that a steady state was reached within 1.5 minutes and showed that the loss of $^{45}CaCl_2$ by diffusion during flow dialysis through the membrane was negligible (~5%).

The calcium binding data were processed according to the theoretical binding models, the Hill Model (Cornish-Bowden and Koshland, 1975; Dahlquist, 1979) or the Klotz - Adair Model (Fletcher et al., 1970) using MacCurveFit program. The quality of the data, however, was not good enough for the determination of macroscopic binding constants. Therefore, the Hill model with fewer parameters was chosen for the binding isotherm analyses. The Hill Model provides information regarding degrees of cooperativity (Hill constant, α), maximum number of Ca^{2+}–binding sites (n), and apparent dissociation constant (K_d (mol/liter)) according to the following equation,

$$r = \frac{nX^{\alpha}}{K_d^{\alpha} + X^{\alpha}}$$

where r and X denote the average number of moles of Ca^{2+} bound per mole of protein and the free Ca^{2+} concentration, respectively (Dahlquist, 1979; Porumb, 1994; Cantor and Schimmel, 1980).

Protein Concentration Determination

Protein concentrations were measured according to Bradford (Bradford, 1976) assay using bovine serum albumin as a standard or by optical density at 280 nm from calculated extinction coefficients for each isoenzyme (Gill and von Hippel, 1989). Protein concentrations determined by both methods were in good agreement.

Protein Kinase Assays and Autophosphorylations

Kinase assays were performed in the presence or absence of calcium as described in Chapter 2, with the following modifications. The assays were carried out in a buffer containing 50 mM HEPES, pH 7.4, 100 mM KCl, 5 mM

$MgCl_2$, 2 mM DTT, 60 μM [γ-^{32}P]ATP (about 500 cpm /pmol), 100 μM syntide-2, and 10 ng of each isoenzyme at 24 ± 2 °C for 6 minutes in the presence of free calcium with varying concentrations from 0.1 μM to 100 μM or in the absence of calcium (10 mM EGTA). At the end of each reaction, 10 μL aliquots were spotted onto precut (about 1.5 cm^2) P-81 phosphocellulose paper and washed 3 times for 1 minute each in 10% phosphoric acid. The remaining radioactivity was measured by liquid scintillation counting. Ca^{2+}/EGTA buffers containing 0.1 μM to 1 μM free calcium in the presence of 0.2 mM free EGTA were prepared according to Tsien and Pozzan (1989). Assay buffers containing free calcium from 5 μM to 100 μM were made by direct dilutions from 0.1 M $CaCl_2$ standard solution purchased from Orion as recommended by Bers et al. (1994). Activity assays of CDPKα using protein substrates were performed in the same assay mixture except that 2 μg/ml of the enzyme was used and 0.5 mg/ml of histone IIIS or 0.2 mg/ml serine acetyltransferase (SAT) (Yoo and Harmon, submitted) substituted peptide substrates.

Autophosphorylation of each isoenzyme were performed in the absence of the substrate at room temperature for 15 minutes. The total 25 μL assay mixtures contained 2.5 μg enzymes in the same buffer used for the activity assays.

Results

Direct Ca^{2+}–Biding Studies

The binding of Ca^{2+} to CDPK isoenzymes measured by flow dialysis is shown in Figure 3-2. The Ca^{2+}–binding curves were analyzed by fitting the curve according to the Hill model as described in Experimental Procedures. The resulting parameters obtained by the Hill model are listed in Table 3-1.

Examination of the Ca^{2+}-binding isotherms of CDPKα and γ revealed rather complex patterns compared to that of CDPKβ.

Table 3-1. Ca^{2+} dissociation and Hill constants of CDPK isoenzymes

enzymes	Kd (μM)	α
CDPKα	44.7 (3)*	1.2 (0.1)
CDPKβ	1.5 (0.1)	1.4 (0.1)
CDPKγ	1.1 (0.2)	0.6

*In parentheses, standard deviations of the fitted values are shown. The parameters were obtained from at least two determinations. The goodness of fit (R^2) for each enzyme (CDPKα, β, and γ) was 0.91, 0.95, and 0.93, respectively.

The Ca^{2+}–binding curve of CDPKβ was well fitted to the Hill model with apparent Kd of 1.5 μM and Hill constant α of ~1.4 suggesting positive cooperativity (Table 3-1). Since soybean CDPKs contain predicted four perfect E-F hand modules, 4 moles of Ca^{2+} were expected to bind per mole of enzyme. But the maximum binding capacity according to the fitted Hill model was slightly lower (n =3.4). Considering the residual bound Ca^{2+} in CDPK samples after passage through Chelex-100 column being < 0.3 mole of Ca^{2+} per mole of enzyme, the n could be increased to 3.7.

The acceptable Ca^{2+} contamination level in the protein sample is suggested to be 0.025-0.1 mole of Ca^{2+} per mole of Ca^{2+}–binding sites (Porumb, 1994). However, the success of decalcification depends on the affinity for Ca^{2+} of a protein of interest and stability after a harsh decalcification method like trichloroacetic acid (TCA) precipitation which can be effectively used for calmodulin (Haiech et al., 1981). For CDPKs, which are chimeric enzymes containing catalytic domain and calmodulin-like domain, only mild and limited treatments may be used to remove contaminating Ca^{2+} while keeping the enzyme active. It is possible that the quality of experiments including the

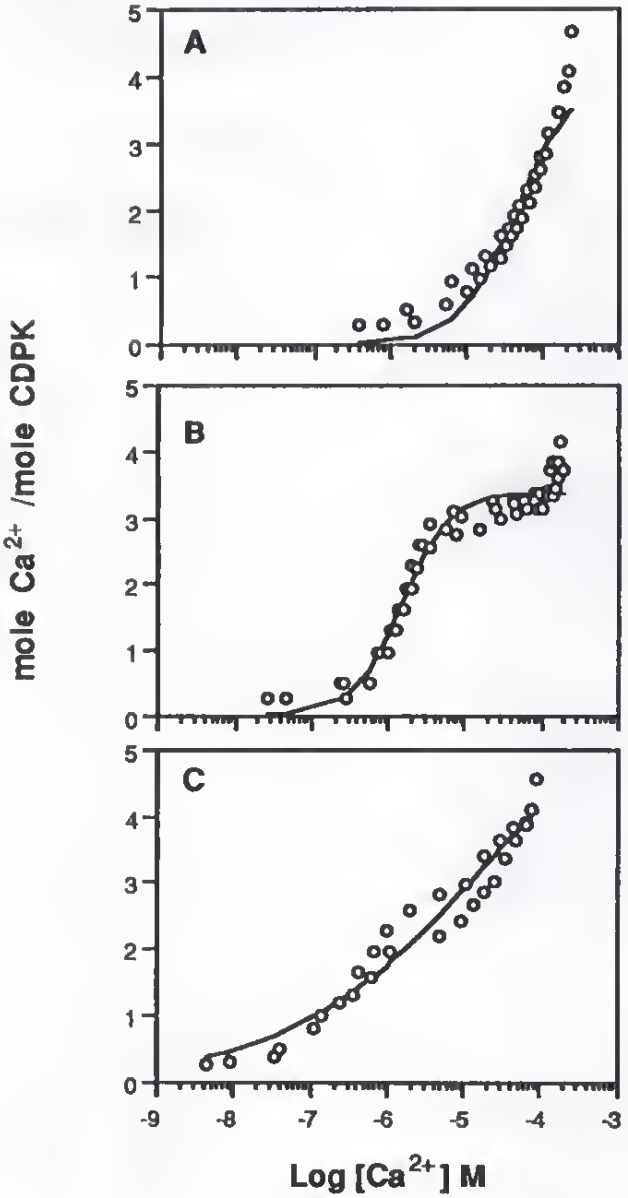

Figure 3-2. Direct Ca^{2+} binding to CDPKα, β, and γ.
Ca^{2+} binding data were obtained from flow dialysis as described in the experimental procedures. The protein concentration was 20 μM in 50 mM HEPES, pH 7.5 containing 100 mM KCl at 25 ± 2 °C.
a) CDPKα ; b) CDPKβ; c) CDPKγ. For CDPKγ, 2 mM DTT was supplemented for the enzyme stability. The average number of moles of Ca^{2+} bound per mole of protein is plotted as a function of free Ca^{2+} without correction of contaminating Ca^{2+}. The solid lines represent computer fitted curve of the binding data (O) according to the Hill model using MacCurveFit program.

protein purity, enzyme stability during dialysis, and accuracy of pipetting may have caused this apparently lower number of binding sites. Errors in protein measurement would also affect the number.

CDPKγ is a highly unstable enzyme (Chapter 2). Full-length CDPKγ precipitated against flow dialysis buffer containing 100 mM KCl during overnight dialysis. The protein remaining in the supernatant of dialysate was recovered and subjected to the flow dialysis. However, the enzyme again precipitated after the first injection of Ca^{2+} was made. Therefore, the N-terminal deletion construct, $CDPK\gamma_{(66-538)}$ was chosen to replace the full-length enzyme for the Ca^{2+}–binding study. The enzyme activity of $CDPK\gamma_{(66-538)}$ was comparable to that of the full-length enzyme but it tolerated the dialysis in the low salt buffer (Chapter 2). Although $CDPK\gamma_{(66-538)}$ could be used for the flow dialysis without precipitation, it was not as stable as the other isoenzymes. GST- $CDPK\gamma_{(66-538)}$ was freshly prepared each time because the enzyme become unstable after overnight storage. To show that Ca^{2+} binding property of CDPKγ was not changed by the deletion of the N-terminus in $CDPK\gamma_{(66-538)}$, the effect of Ca^{2+} concentration on the kinase activity was examined. The result confirmed that the response was identical to that of the full-length enzyme (data not shown).

The Ca^{2+}–binding data of $CDPK\gamma_{(66-538)}$ was fitted to the Hill model by setting n =4 due to the absence of apparent saturation of Ca^{2+} binding to the enzyme. The resulting Kd and Hill constant α were ~1 μM and ~0.5 μM, respectively. The Klotz-Adair model (Klotz, 1983; Porumb, 1994) was better in the curve fitting for CDPKγ. The resulting macroscopic dissociation constants, however, showed high values of error, which made the result of the analysis difficult to interpret. These results may imply that the behavior

of Ca^{2+} binding of CDPKγ is complicated and requires further intensive analyses with better data from flow dialysis or other methods.

CDPKα also showed no apparent saturation of Ca^{2+} binding (Figure 3-2a). The Ca^{2+}–binding data fitting for CDPKα was undertaken according to the Hill model but the parameters were obtained by setting n to 4 as for CDPKγ above. CDPKα had a higher Kd (~45 μM) relative to the other CDPK isoenzymes and nonspecific binding was observed at around 100 μM free Ca^{2+} making hard to predict the maximum number of binding with the Hill Model without setting n to 4. Nonspecific binding of Ca^{2+} was detected for all three enzymes. This may be attributed to the limitation of flow dialysis method (Kakalis et al., 1995; Porumb, 1994). The examination of the Ca^{2+}–binding curve of CDPKα suggested that it is similar to that of PfCDPK (Zhao et al., 1994). The Ca^{2+}–binding to PfCDPK was measured by equilibrium dialysis and the resulting Kd and n were reported to be 80 μM and 3.9 respectively.

Analysis of Ca^{2+}–Dependent Kinase Activity of CDPKs

To elucidate how the Ca^{2+}-binding property of each CDPK isoenzyme affects its kinase activity, changes of kinase activity in response to various Ca^{2+} concentration were investigated (Figure 3-3). The Ca^{2+} concentrations for half maximal kinase activity ($K_{0.5}$) for CDPKβ and γ were 0.5 and 1 μM, respectively. These values were closely correlated to their Ca^{2+} binding behavior, which suggested that 1 or 2 calcium ions bound per mole of each enzyme may be sufficient for the half maximal enzyme activity. The kinase activities of these two enzymes were saturated at a similar concentration, ~ 5 μM. At this Ca^{2+} concentration, Ca^{2+}–binding to CDPKβ was also saturated (Figure 3-2b) implying that occupation of four Ca^{2+}–binding sites was required for the maximum activity of CDPKβ.

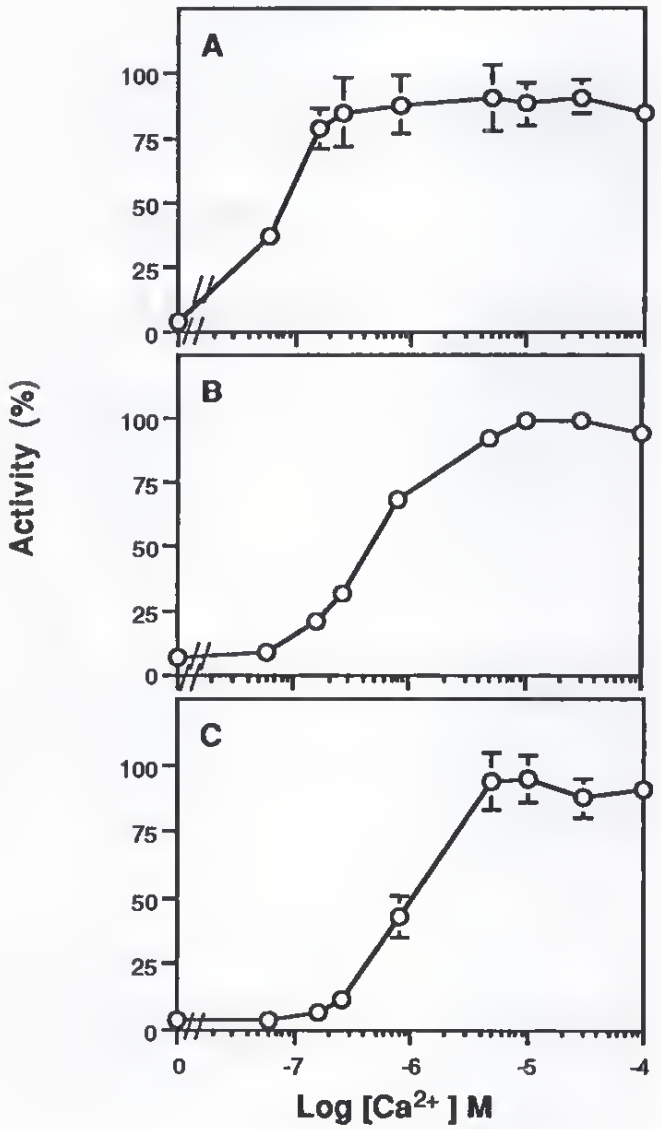

Figure 3-3. The effect of Ca^{2+} on kinase activity of CDPKs.
Enzyme activity assays were performed in the buffers with varied free Ca^{2+} concentrations or in the presence of 1mM EGTA (indicated as zero Ca^{2+} on abscissa) using syntide-2 as a peptide substrate as described in the Experimental Procedures. Standard deviations are shown as vertical bars from at least two independent experiments.
a) CDPKα; b) CDPKβ; c) CDPKγ.

The $K_{0.5}$ of CDPKα was ~0.1 μM (Figure 3-3a). This result was very surprising because the Kd for Ca^{2+} of CDPKα was the highest (~45 μM) of the three isoenzymes and the stoichiometric Ca^{2+} binding of CDPKα occurred at calcium concentration above 10 μM (Figure 3-2a). Moreover, the saturation of kinase activity of CDPKα appeared to be only about 0.3 μM. This value is also far below the concentration of stoichiometric Ca^{2+} binding to the enzyme and about ten-fold lower than that of the other two enzymes.

Ca^{2+}–Binding Studies of CDPKα in Various Conditions Resembling the Enzyme Assay Mixture

The Ca^{2+}–binding behavior of CDPKα was further examined in order to understand what caused the apparent change in Ca^{2+} sensitivity of the enzyme in the activity assay. Since there were additional components in the assay mixture such as ATP, $MgCl_2$, DTT, and a peptide substrate, syntide-2, the effects of these components on the Kd of CDPKα were inspected. First, assay components that were not present in the flow dialysis buffer were added alone or as a combination to the dialysis buffer and the flow dialysis was carried out in the same manner as described in Experimental Procedures. $MgCl_2$ and DTT did not show any effect on the Ca^{2+}–binding of CDPKα (data not shown). However, as shown in Figure 3-4, quite interesting results were obtained in the presence of peptide substrate, syntide-2, and ATP. The Kd for Ca^{2+} of CDPKα was markedly reduced to ~1 μM from ~45 μM. This striking shift of Kd apparently resembled the influence on the Kd for Ca^{2+} of calmodulin in the presence of a peptide containing the calmodulin binding site (Porumb, 1994; Yazawa, 1992). The enhanced Ca^{2+}-binding affinity of CDPKα was mainly caused by the presence of syntide-2. The presence of ATP

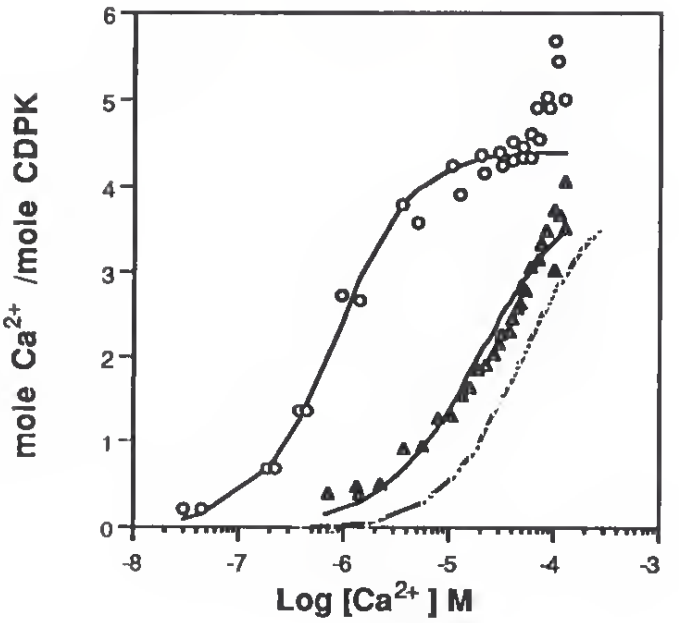

Figure 3-4. Ca^{2+}–binding studies of CDPKα under various conditions. Flow dialysis was performed to examine the effect of syntide-2 and (or) ATP on Ca^{2+}–binding property of CDPKα. The sample in the upper chamber contained 7 μM of CDPKα and 1.5 μM of $^{45}CaCl_2$ in a dialysis buffer (50 mM HEPES, pH 7.5, 100 mM KCl, 5 mM $MgCl_2$, and 60 μM ATP). The fitted curves of Ca^{2+}–binding data in the presence (o) or the absence (Δ) of 100 μM syntide-2 are shown. The control experiment (—) performed in a dialysis buffer containing 50 mM HEPES and 100 mM KCl is from Figure 3-2a.

alone slightly affected the Kd (Figure 3-4) but syntide-2 alone could cause the shift even more dramatically than when combined with ATP (data not shown).

Syntide 2 is the best substrate peptide tested for all three soybean CDPKs (Chapter 2). It was asked whether other peptides which are not good substrates for CDPKα but contain the motif, basic-X-X-ser/thr could shift the Kd. Including H1-7 (RRKASGP) or skeletal muscle myosin light chain kinase substrate (AKRPQRATSNVFS) in the Ca^{2+}-binding experiments resulted in the Kd shift similar to that caused by syntide-2 (data not shown). It can be speculated from these results that these peptides are capable to bind the enzyme as well as syntide-2 and cause the shift of Kd for Ca^{2+} but the phosphorylation by CDPKα is not favorable. It was concluded that the low $K_{0.5}$ of CDPKα as shown in Figure 3-3a is not an experimental anomaly but caused by syntide-2. If this titration curve (Figure 3-3a) is compared directly to the binding curve in the presence of syntide-2 shown in Figure 3-4, it is clear that kinase activity is well correlated to that of Ca^{2+}–binding behavior in the same condition as activity assay.

$K_{0.5}$ of CDPKα in the Presence of Protein Substrates

The observation that Kd for Ca^{2+} of CDPKα could be substantially decreased in the presence of syntide-2 raised a question whether the phosphorylation by CDPKα occurs always at low Ca^{2+} regardless of its substrate. Two protein substrates, histone IIIS and SAT were chosen for the further examination regarding this question. Histone IIIS is not a favorable substrate of CDPKα but the phosphorylation occured sufficiently to detect the effect of Ca^{2+} on the kinase activity. Interestingly, $K_{0.5}$ for histone IIIS phosphorylation by CDPKα turned out to be about 4 μM (Figure 3-5) which is forty-fold higher than that

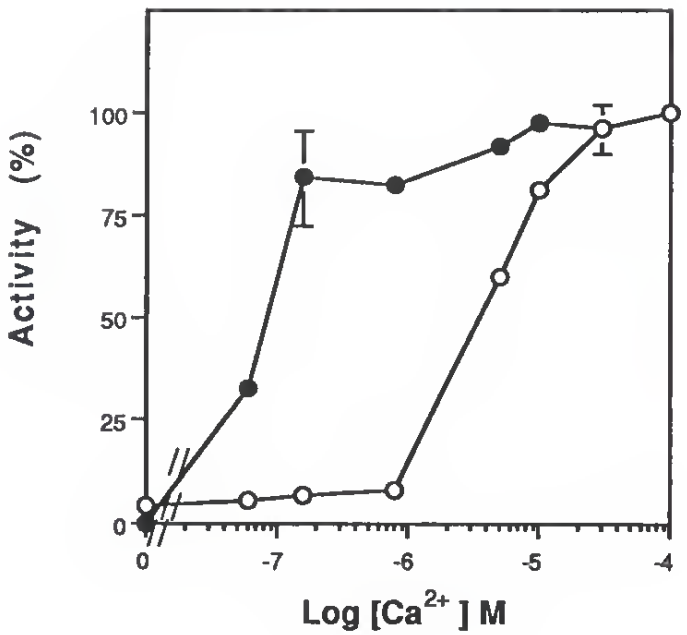

Figure 3-5. Changes in $K_{0.5}$ of CDPKα by different protein substrates as a function of free Ca^{2+} concentration.
The effect of different protein substrates on the activity of CDPKα in respense to Ca^{2+} concentration was determined. The assays were performed as described in Experimental procedures by adding 0.5 mg/ml histone IIIS (o) or 0.2 mg/ml SAT (•) as a substrate.

for syntide-2 phosphorylation ($K_{0.5}$ ~0.1 μM). Saturation of histone IIIS phosphorylation occurred at ~30 μM. These results appear to be consistent with the Ca^{2+}–binding behavior observed in the absence of syntide-2 (Figure 3-2A and Table 3-1) and imply that Kd for Ca^{2+} of CDPKα may depend on its substrate. These results also confirm the validity of Ca^{2+}–binding data obtained in the absence of a substrate peptide.

Since it was observed that H1-7 or skeletal muscle myosin light chain kinase substrate were able to change Kd for Ca^{2+} of CDPKα but were hardly phosphorylated by the enzyme, it was asked whether the presence of these peptides may affect the $K_{0.5}$ for histone IIIS phosphorylation by CDPKα. When these peptides were added to the assay mixture, however, the $K_{0.5}$ for histone IIIS phosphorylation was not affected (data not shown). These results imply the possibility that these peptides were unable to bind the enzyme in the presence of histone IIIS.

A more remarkable observation was made when SAT was used as a protein substrate for CDPKα. SAT is a novel *in vitro* substrate of CDPKs identified by interaction cloning (Yoo and Harmon, submitted). As shown in Figure 3-5, $K_{0.5}$ for SAT is ~0.1 μM, which is identical to that for syntide-2. These results propose that certain substrates can induce the high affinity for Ca^{2+} of CDPKα despite the intrinsically low affinity for Ca^{2+} of CDPKα. If this is true *in vivo*, it could be a mechanism of regulating the activity of CDPKα in response to different Ca^{2+} signals and for the selection of the specific substrate.

Autophosphorylation of CDPKs in Response to Ca^{2+}

The effects of Ca^{2+} on autophosphorylation of CDPKs were investigated. Maximum autophosphorylation of CDPKα was observed at very low Ca^{2+} concentration (~0.3 μM) (Figure 3-6). But the autophosphorylation is Ca^{2+}–

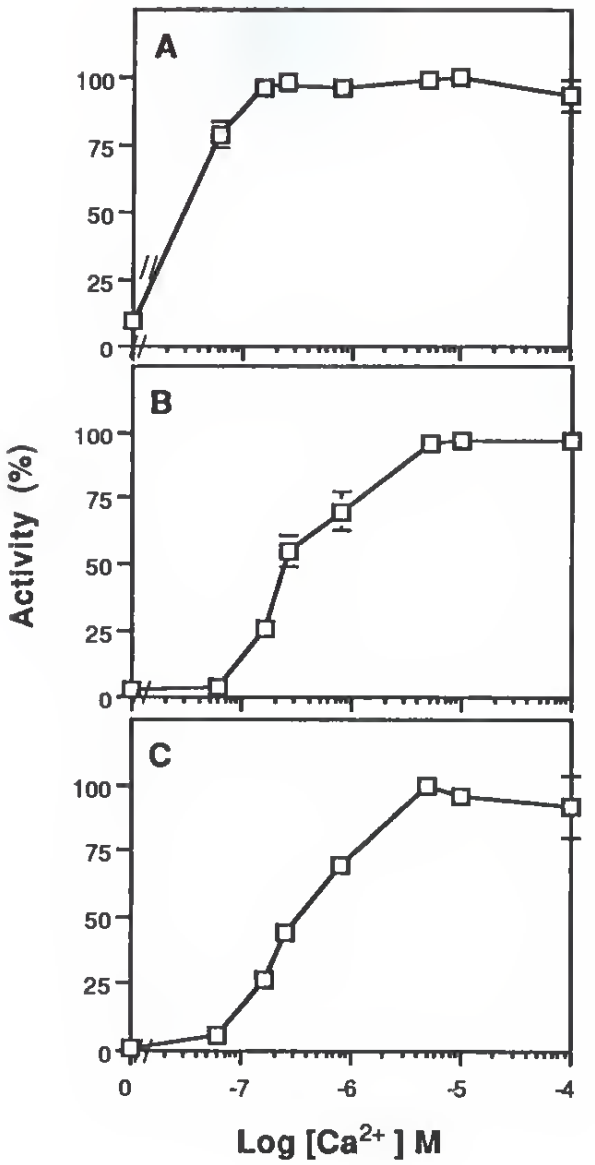

Figure 3-6. The effect of Ca^{2+} on autophosphorylation of CDPKs.
a) Autophosphorylation of CDPKα, b) β, and c) γ was performed as described in Experimental Procedures.

dependent because only basal level (less than 10 % of maximum autophosphorylation activity) of autophosphorylation was observed in the presence of 1 mM EGTA. For CDPKβ and γ, ~5 μM of free Ca^{2+} was required for the saturation of the autophosphorylation. It is intriguing that autophosphorylation of CDPKα is highly sensitive to low Ca^{2+} because high affinity Ca^{2+} binding site(s) was not detected when a substrate is absent. Moreover, the contaminating Ca^{2+} was measured to be less than 0.3 mole of Ca^{2+} per mole of enzyme in the flow dialysis buffer. Therefore, if there is high affinity Ca^{2+} binding site(s) in CDPKα the Kd for that site(s) may be lower than the Ca^{2+} contamination level in the flow dialysis but probably higher than that of EGTA (~60 nM under the assay conditions).

Discussion

CDPK Isoforms Differ in Their Ca^{2+}-Binding Properties

The Ca^{2+}-binding properties of three soybean CDPK isoforms were investigated to test the hypothesis that each CDPK isoenzyme may be able to transduce distinct Ca^{2+} signals upon stimuli through its altered Ca^{2+}-sensitivity. The results clearly provided the biochemical evidence that each isoenzyme differs in Ca^{2+}-binding properties. Although there were some experimental difficulties due to the enzyme stability during purification and flow dialysis, the Ca^{2+}-binding data obtained with recombinant CDPKs were reproducible.

Fitting and interpreting data according to the simple ligand binding models were not straightforward though the correlating coefficients were high. It is possible that the data quality was not high enough and/or the Ca^{+}-binding behavior of CDPKs was too complicated to be predicted by the

theoretical binding models. For example, Hill constant α for CDPKγ was predicted to be 0.6 (Table 3-1). Hill constant $\alpha < 1$ is interpreted as anticooperativity (Porumb et al., 1994). Zhao et al. (1994) have ascribed the quality of their Ca^{+}-binding data for PfCDPK to the difficulty of analysis with theoretical binding model. Fitting the Ca^{2+}-binding data of CDPKβ with Hill model, however, was relatively satisfactory. The result suggested positive cooperative binding. The saturation of Ca^{2+}-binding to CDPKβ was observed at ~10 μM of free Ca^{2+} unlike CDPKα and γ. The nonspecific binding of Ca^{2+} to CDPKα and γ at and above 100 μM was a probable limiting factor to get saturation of these enzymes.

The mean Kds of CDPKβ and γ were similar (1.5 and 1.1 μM, respectively), though the shape of Ca^{2+}-binding isotherms of these enzymes were distinctive (Figure 3-2). These values of Kd are in good accordance with that of calmodulin (1.5 to 5 μM). CDPKα, however, had a much higher Kd of about 45 μM. The Kd for Ca^{2+} of CDPKα was comparable to the Kd of PfCDPK (Zhao et al., 1994). Since the amino acid sequence comparison showed that CDPKα and β are closer to each other than each of them to CDPKγ, the result was unexpected. Moreover, the sequence of PfCPK is highly diverged not only from the sequence of CDPKα but also from the sequences of any of CDPKs in plants. The sequence analysis of CLD domains of three soybean CDPKs and calmodulin showed that the sequence identity between calmodulin and soybean CDPKs were ~40% but the amphiphilic characteristics of calmodulin was highly conserved in CLD domains (data not shown). In addition, quite a few residues in calmodulin known to interact with a target peptide derived from smMLCK (Meador et al., 1993) were consistenly conserved in the CLD domains of the three CDPKs. These results from the sequence analysis suggested that it is difficult to point out which

residues are responsible for the differences in Ca^{2+}-binding properties, especially, between CDPKα and β. It will be necessary to learn the actual structures of CDPKs and the relationships between CLD and autoinhibitory domain for the elucidation of the differences in Ca^{2+}-binding properties among CDPKs.

CDPKα Shows High Ca^{2+} Sensitivity in the Presence of Certain Substrates

The Kd and $K_{0.5}$ for Ca^{2+} of CDPKβ and γ were almost equivalent (Table 3-1 and 3-2). In contrast, the $K_{0.5}$ of CDPKα was over 400-fold lower than its Kd. Further examination showed that the Kd for Ca^{2+} of CDPKα decreased to a value near the Kd when syntide-2 was included in the direct Ca^{2+}-binding experiments. Also it was observed that autophosphorylation of CDPKα was Ca^{2+}-dependent but its requirement for free Ca^{2+} was very low (< 0.3 μM).

Table 3-2. $K_{0.5}$s for Ca^{2+} of CDPKα, β, and γ with various substrates.

	Syntide-2	SAT	Histone IIIS
Enzyme		$K_{0.5}$ (μM)	
CDPKα	0.1	0.1	4
CDPKβ	0.5	nd[a]	nd[a]
CDPKγ	1	nd[a]	nd[a]

[a]nd, not determined.

CDPKα may contain at least one high affinity Ca^{2+}-binding site which was not detectable in the flow dialysis experiment. Since the contaminating Ca^{2+} concentration in the buffer was ~0.3 μM, it can be speculated that the high affinity Ca^{2+}-binding site may have been filled prior the initiation of Ca^{2+}-titration, if the Kd of this site was lower than 0.3 μM. The presence of

high affinity Ca^{2+}-binding site in CDPKα could be tested in an alternative method to the flow dialysis. Determination of Ca^{2+} dissociation constants between 10^{-7} and 10^{-9} M has been obtained from Ca^{2+} titrations of the protein in the presence of chromophoric Ca^{2+} chelators such as Quin2, BAPTA (bis(2-aminophenoxy)ethane-*N*,*N*,*N'*,*N'*-tetraacetic acid), or Fluo-3 (Eberhard and Erne, 1991, 1994; Linse et al., 1991; Waltersson et al., 1993). The binding of Ca^{2+} to these compounds was monitored by the absorbance at the specific wave length.

The enhancement of Ca^{2+}-binding affinity of CDPKα in the presence of syntide-2 resembled that of calmodulin induced by the complex formation with its numerous target proteins or peptides such as MLCK, cyclic nucleotide phosphodiesterase (Olwin and Storm, 1985), caldesmon, mastoparan (Yazawa et al., 1987), and calmodulin binding peptides derived from plasma membrane calcium pump (Yazawa et al., 1992) and calcineurin (Stemmer and Klee, 1994). CDPK contains an autoinhibitory domain that acts as a pseudosubstrate (Harmon et al., 1994; Harper et al., 1994; Yoo and Harmon, 1996). The binding of Ca^{2+} to calmodulin-like domain is proposed to release the autoinhibitory domain from the catalytic domain thus activating the enzyme. The enzyme activity of CDPKα is only basal level in the presence of EGTA. If it is presumed that CDPKα has a high affinity Ca^{2+}-binding site and the binding of Ca^{2+} to this site gives rise to a conformation which syntide-2 can bind to the catalytic domain resulting in freeing CDL binding site, the CLD could form a complex with the CLD binding site. This complex formation might have driven the shift of Ca^{2+}-binding affinity, by analogy to the enhanced Ca^{2+}-binding affinity of calmodulin by forming a complex with a target peptide. The fact that the enzyme activity of CDPKα was minimal in

the absence of Ca^{2+} could be interpreted that the Kd of the high affinity Ca^{2+}-binding site may be lower than that of EGTA.

It was intriguing that CDPKα showed comparably low $K_{0.5}$ for Ca^{2+} in phosphorylating a novel *in vitro* protein substrate, SAT, and syntide-2, while the $K_{0.5}$ for Ca^{2+} was much higher in the presence of another protein substrate histone IIIS. The inducible Ca^{2+} affinity of CDPKα could serve as another mechanism controlling the CDPK activity *in vivo* for selecting specific substrate(s) on top of the apparently narrow substrate preference of the enzyme. Some substrate might be phosphorylated only at high free Ca^{2+} concentration in the cell if they are not effective in changing the Kd for Ca^{2+} of CDPKα. It may be possible that local Ca^{2+} concentration rises high enough in certain cases so that CDPKα become active to phosphorylate such substrates. In this case CDPKα may need to be localized in vicinity of the Ca^{2+} sources. The presence and the availability of the substrate that are capable to enhance Ca^{2+}-binding affinity of CDPKα may be an important factor for the phosphorylation of those substrates because the resting level of free Ca^{2+} in the cell may be high enough to activate the enzyme. It will require further research to test these speculations but will provide much insight into this likely novel regulatory mechanism of CDPKα.

CHAPTER 4
SUMMARY AND CONCLUSIONS

Summary of Results

Two new CDPK isoforms were isolated from a cDNA library made from soybean cell suspension culture. These two isoforms were designated CDPKβ and CDPKγ. They contained highly conserved structures composed of a catalytic, a junction, and a calmodulin-like domain like those in the structure of CDPKα. CDPKβ contains 490 amino acid residues and has a predicted molecular mass of 55 kD. CDPKγ has higher molecular mass of 60 kD composed of 538 amino acid residues. CDPKβ and CDPKγ showed 76% and 58% overall amino acid sequence identity, respectively, to CDPKα. CDPKγ is unique in that it has an 8 amino acid insertion in the calmodulin-like domain. It also has a relatively long N-terminal sequence prior to the catalytic domain compared to the other two CDPKs. The N-terminal sequence of CDPKγ did not show any similarity to the known protein motifs. Deleting the N-terminal sequence had no effect on the enzyme activity.

Northern blot analysis showed that all three were expressed constitutively either in cell culture or mature soybean plants. However, the relative distribution of each transcript was different. mRNA of CDPKα was predominantly expressed in three day-old cell culture and that of each CDPKβ and γ were highly expressed in leaf and root, respectively. Whether these apparent differences in mRNA expression are closely related to their specific enzyme function remains to be investigated.

Recombinant CDPKs fused to GST were expressed as active enzymes in *E. coli*. They were highly purified by affinity and anion exchange chromatographies. Each CDPK isoform showed different biochemical properties. Unlike CDPKα and β, CDPKγ was an unstable enzyme. It lost substantial enzyme activity when stored in 50% glycerol at -80 °C, precipitated in low salt buffer, and required DTT for enzyme activity. The CDPKs had broad substrate specificity, but they differed in the fold-activation by Ca^{2+} and in K_m and V_{max} when syntide-2 and smooth muscle myosin light chain kinase substrate peptide were used as substrates. All three CDPKs were inhibited by the general protein kinase inhibitors, K-252a and staurosporine, with higher IC_{50}s compared to those of other kinases. Interestingly, calphostin C known as protein kinase C-specific inhibitor, was a potent inhibitor of CDPKs.

Examination of Ca^{2+}-binding properties using flow dialysis further demonstrated that CDPK isoforms are not redundant enzymes. The binding isotherm of each enzyme was different, and the Kd for Ca^{2+} of CDPKα, β, and γ was ~45, 1, and 1.5 μM, respectively. $K_{0.5}$ for Ca^{2+} of CDPKs ranged from 0.1 to 1 μM for the syntide-2 phosphorylation. Notably CDPKα showed enhanced Ca^{2+}-binding affinity (Kd of ~1 μM) in the presence of syntide-2 and lower $K_{0.5}$ for phosphorylating syntide-2 or SAT than for phosphorylating histone IIIS.

Conclusions

CDPK isoforms from soybean shared high amino acid sequence identity (50-80%) but they were distinct enzymes. The expression patterns of mRNAs, biochemical properties, substrate specificities, and Ca^{2+}-binding properties were overlapping but not completely redundant. The broad substrate specificities of CDPKs suggested that they are multifunctional protein kinases.

The Kd for Ca^{2+} of CDPKα was significantly higher than that of the other two enzymes. Its Ca^{2+}-binding affinity, however, was increased in the presence of some substrates. All together, the results strongly support the hypothesis that CDPK isoenzymes may have role(s) in mediating different Ca^{2+} signal leading to specific physiological responses.

REFERENCES

Allan, A.C., Fricker, M., Ward, J., Beale, M.H., and Trewavas, A.J. (1994). Two transduction pathways mediate rapid effects of abscisic acid in *Commelina* guard cells. Plant Cell **37**, 840-846.

Allbritton, N.L., Meyer, T., and Stryer, L. (1992). Range of messenger action of calcium ion and inositol 1, 4, 5-triphosphate. Science **258**, 1812-1815.

Allen, G.J., and Sanders, D. (1994). Two voltage-gated, calcium release channels coreside in the vacuolar membrane of broad bean guard cells. Plant Cell **6**, 685-694.

Allen, G.J., Muir, S. R., and Sanders, D. (1995). Release of Ca^{2+} from individual plant vacuoles by both $InsP_3$ and cyclic ADP-ribose. Science **268**, 735-737.

Allen, G.J., and Sanders, D. (1995). Calcineurin, a type 2B protein phosphatase, modulates the Ca^{2+}-permeable slow vacuolar ion channel of stomatal guard cells. Plant Cell **7**, 1473-1483.

Armstrong, F. and Blatt, M.R. (1995). Evidence for K^+ channel control in *Vicia* guard cells coupled by G-proteins to a 7TMS receptor mimetic. Plant J. **8**, 187-198.

Assmann, S.M. (1996). Guard cell G proteins. Trends Plant Sci. **1**, 74-75.

Babu, Y.S., Bugg, C.E., and Cook, W.J. (1988). Structure of calmodulin refined at 2.2 A resolution. J. Mol. Biol. **204**, 191-204.

Babu, Y.S., Sack, J.S., Greenbough, T.C., Bugg, C.E., Means, A.R., and Cook, W.J. (1985). The three-dimensional structure of calmodulin. Nature **315**, 37-40.

Bachmann, M., Huber, J. L., Cambell, W.H., Yoo, B.-C., Harmon, A.C., and Huber, S.C. (1996). Identification of the major regulatory phosphorylation site in spinach leaf nitrate reductase and its phosphorylation by a Ca^{2+}-dependent protein kinase in vitro. Plant Cell **8**, 505-517.

Beckingham, K. (1991). Use of site-directed mutations in the individual Ca^{2+}-binding sites of calmodulin to examine Ca^{2+}-induced conformational changes. J. Biol. Chem. **266**, 6027-6030.

Berridge, M.J. (1993). Inositol trisphosphate and calcium signalling. Nature **361**, 315-325.

Berridge, M.J., and Dupont, G. (1994). Spatial and temporal signalling by calcium. Curr. Biol. 6, 267-274.

Bers, D.M., Patton, C.W., and Nuccitelli, R. (1994). A Practical guide to the preparation of Ca^{2+} buffers. Meth. Cell Biol. **40**, 3-29.

Bethke, P.C. and Jones, R.L. (1994). Ca^{2+}-calmodulin modulates ion channel activity in storage protein vacuoles of barley aleurone cells. Plant Cell 6, 277-285.

Biswas, S., Dalal, B., Sen. M., and Biswas, B.B. (1995) Receptor for *myo*-inositol trisphosphate from the microsomal fraction of *Vigna radiata*. Biochem. J. **306**, 631-636.

Blinks, J.R. (1989). The use of calcium-regulated photoproteins as intracellular Ca^{2+} indicators. Meth. Enzymol. **172**, 164-203.

Bootman, M.D., and Berridge, M. (1995). The elemental principles of calcium signaling. Cell 83, 675-678.

Borochov-Neori, H., and Harmon, A.C. (1993). Identification and characterization of CDPK-related proteins in the soybean cell plasma membrane. In 12th Annual Missouri Plant Biochem. Mol. Biol. and Physiol. Symposium, University of Missouri-Columbia, Missouri, pp. 151.

Botella, J.R., Arteca, J.M., Somodevilla, M., and Arteca, R.N. (1996). Calcium-dependent protein kinase gene expression in response to physical and chemical stimuli in mung bean (*Vigna radiata*). Plant Mol. Biol. **30**, 1129-1137.

Bowler, C., Neuhaus, G., Yamagata, H., and Chua, N.-H. (1994). Cyclic GMP and calcium mediate phytochrome phototransduction. Cell **77**, 73-81.

Braam, J. (1992). Regulated expression of the calmodulin-related *TCH* genes in cultured *Arabidopsis* cells: Induction by calcium and heat shock. Proc. Natl. Acad. Sci. USA 89, 3213-3216.

Braam, J. and Davis, R.W. (1990). Rain-, wind-, and touch-induced expression of calmodulin and calmodulin-related genes in *Arabidopsis*. Cell **60**, 357-364.

Bradford, M.M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. **72**, 248-254.

Breviario, D., Morello, L., and Giani, S. (1995). Molecular cloning of two novel rice cDNA sequences encoding putative calcium-dependent protein kinases. Plant Mol. Biol. **27**, 953-967.

Brown, T. (1992). Analysis of RNA by Northern and slot blot hybridization. In Current Protocols in Molecular Biology, Ausubel, F.M., Brent, R., Kingston, R.E., Moore, D.D., Seidman, J.G., Smith, J.A. and Struhl, K. eds. (New York: Green/Wiley-InterScience), pp. 4.9.1-4.9.14.

Bush, D. (1993). Regulation of cytosolic calcium in plants. Plant Physiol. **103**, 7-13.

Bush, D.S. (1995). Calcium regulation in plant cells and its role in signaling. Annu. Rev. Plant Physiol. Plant Mol. Biol. **46**, 95-122.

Bush, D.S. (1996). Effects of gibberellic acid and environmental factors on cytosolic calcium in wheat aleurone cells. Planta **199**, 89-99.

Cantor, C.R., and Schimmel, P.R. 1980. Biophysical Chemistry. The behavior of biological macromolecules. Freeman, San Francisco.

Cheung, W.Y. (1980). Calmodulin plays a pivotal role in cellular regulation. Science **207**, 19-27.

Chomczynski, P. (1992). One-hour downward alkaline capillary transfer for blotting of DNA and RNA. Anal. Biochem. **201**, 134-139.

Clapham, D.E. (1993). A mysterious new influx factor? Nature **364**, 763-764.

Clapham, D.E. (1995). Calcium signaling. Cell **80**, 259-268.

Colowick, S.P., and Womack, F.C. (1969). Binding of diffusible molecules by macromolecules: rapid measurement by rate of dialysis. J. Biol. Chem. **244**, 774-777.

Cornish-Bowden, A., and Koshland, D.E.J. (1975). Diagnostic uses of the Hill (Logit and Nernst) plots. J. Mol. Biol. **95**, 201-212.

Cox, J.A., Malnoe, A., and Stein, E. (1981). Regulation of brain cyclic nucleotide phosphodiesterase by calmodulin. J. Biol. Chem. **256,** 3218-3222.

Cramer, G.R., and Jones, R.L. (1996). Osmotic stress and abscisic acid reduce cytosolic calcium activities in roots of *Arabidopsis thaliana*. Plant Cell Environ. **19,** 1291-1298.

Crouch, T.H., and Klee, C.B. (1980). Positive cooperative binding of calcium to bovine brain calmodulin. Biochemistry **19,** 3693-3698.

Dahlquist, F.W. (1979). The meaning of Scatchard and Hill plots. Meth. Enzymol. **48,** 270-299.

Dekker, L.V., and Parker, P.J. (1994). Protein kinase C-a question of specificity. Trends Biochem. Sci. **19,** 73-77.

Eberhard, M., and Erne, P. (1991). Analysis of Calcium binding to α-lactalbumin using a fluorescent calcium indicator. Eur. J. Biochem. **202,** 1333-1338.

Eberhard, M., and Erne, P. (1994). Calcium and magnesium binding to rat parvalbumin. Eur. J. Biochem. **222,** 21-26.

Ehrhardt, D.W., Wais, R., and Long, S.R. (1996). Calcium spiking in plant root hairs responding to *Rhizobium* nodulation signals. Cell **85,** 673-681.

Estruch, J.J., Kadwell, S., Merlin, E., and Crossland, L. (1994). Cloning and characterization of a maize pollen-specific calcium-dependent calmodulin-independent protein kinase. Proc. Natl. Acad. Sci. USA **91,** 8837-3341.

Fairley-Grenot, K. and Assmann, S.M. (1991). Evidence for G-protein regulation of inward K channel current in guard cells of fava beans. Plant Cell **3,** 1037-1044.

Fallon, K., Shaklock, P.S., and Trewavas, A. J. (1993). Detection in vivo of very rapid red light-induced calcium-sensitive protein phosphorylation in etiolated wheat (*Triticum aestivum*) leaf protoplasts. Plant Physiol. **101,** 1039-1045.

Farmer, P.K., and Choi, J.H. (1995). Expression and potential myristoylation of a calcium-dependent protein-kinase. J. Cell. Biochem. Suppl. **21A,** 507.

Felle, H. (1988). Auxin causes oscillations of cytosolic free calcium and pH in *Zea mays* coleoptiles. Planta **174**, 495-499.

Felle, H., Tretyn, A., and Wagner, G. (1992). The role of the plasma-membrane Ca^{2+}-ATPase in Ca^{2+} homeostasis in *Sinapis alba* root hairs. Planta **188**, 306-313.

Finn, B. R., and Forsen, S. (1995). The evolving model of calmodulin structure, function and activation. Structure **3**, 7-11.

Fletcher, J.E., Spector, A.A., and Ashbrook, D. (1970). Analysis of macromolecule-ligand binding by determination of stepwise equilibrium constants. Biochemistry **9**, 4580-4587.

Franklin-Tong, V.E., Drobak, B., Allan, A., Watkins, P.A.C., and Trewavas, A.J. (1996). Growth of pollen tubes of *Papaver rhoeas* is regulated by slow-moving calcium wave propagated by inositol 1,4,5-triphosphate. Plant Cell **8**, 1305-1321.

Frankin-Tong, V.E., Ridie, J.P., and Franklin, F.C.H. (1995). Recombinant stigmatic self-incompatibility (S-) protein elicits a Ca^{2+} transient in pollen of *Papaver rhoeas*. Plant J. **8**, 299-307.

Fyfe, L.B. and Roberts, H.R. (1995). Regulation of cellular calcium through signaling cross-talk involves an intricate interplay between the actions of receptors, G-proteins, and second messengers. FASEB J. **9**, 1297-1303.

Gehring, C.A., Irving, H.R., and Parish, R.W. (1990). Effects of auxin and abscisic acid on cytosolic calcium and pH in plant cells. Proc. Nat. Acad. Sci. USA **87**, 9645-9649.

Gehring, C.A., Williams, D.A., Cody, S.H., and Parish, R.W. (1990). Phototropism and geotropism in maize coleoptiles are partially correlated with increases in cytosolic free calcium. Nature **345**, 528-530.

Geiser, J.R., van Tuinin, D., Brockerhoff, S. E., Neff, M.M., and Davis, T.N. (1991). Can calmodulin function without binding calcium? Cell **65**, 949-959.

Ghosh, A., and Greenberg, M.E. (1995). Calcium signaling in neurons: molecular mechanisms and cellular consequences. Science **268**, 239-246.

Gill, S.C., and von Hippel, P.H. (1989). Calculation of protein extinction coefficients from amino acid sequence data. Anal. Biochem. **182**, 319-326.

Gilroy, S. (1996). Signal transduction in barley aleurone protoplasts is calcium dependent and independent. Plant Cell **8**, 2193-2209.

Gilroy, S., Bethke, P.C., and Jones, R. (1993). Calcium homeostasis in plants. J. Cell Sci. **106**, 453-462.

Gilroy, S., Fricker, M.D., Read, N.D., and Trewavas, A.J. (1991). Role of calcium in signal transduction of *Commelina* guard cells. Plant Cell **3**, 333-344.

Gilroy, S. and **Jones, R.L.** (1992). Gibberellic acid and abscisic acid coordinately regulate cytoplasmic calcium and secretory activity in barley aleurone protoplasts. Proc. Natl. Acad. Sci. USA 89, 3591-3595.

Gilroy, S. and **Jones, R.L.** (1993). Calmodulin stimulation of unidirectional calcium uptake by the endoplasmic reticulum of barley aleurone. Planta **190**, 289-296.

Gilroy, S., Read, N.D., and Trewavas, A.J. (1990). Elevation of cytoplasmic calcium by caged calcium or caged inositol triphosphate initiates stomatal closure. Nature **346**, 769-771.

Giraudat, J., Parcy, F. Bertauche, N., Gosti, F., Leung, J., Moris, P.-C., Bouvier-Durand, M., and Vartanian, N. (1994). Current advances in abscisic acid action and signalling. Plant Mol. Biol. **26**, 1557-1577.

Guan, K., and Dixon, J.E. (1991). Eukaryotic proteins expressed in *Escherichia coli*: an improved thrombin cleavage and purification procedure of fusion proteins with glutathione S-transferase. Anal. Biochem. **192**, 262-267.

Gunderson, R.E. and **Nelson, D.L.** (1987). A novel Ca^{2+}-dependent protein kinase from *Paramecium tetraurelia*. J. Biol. Chem. **262**, 4602-4609.

Haiech, J., Kilhoffers, M.-C., Lukas, T.J., Craig, T.A., Roberts, D.M., and Watterson, D.M. (1991). Restoration of the calcium binding activity of mutant calmodulins toward normal by the presence of a calmodulin binding structure. J. Biol. Chem. **266**, 3427-3431.

Haiech, J., Klee, C.B., and Demaille, J.G. (1981). Effects of cations on affinity of calmodulin for calcium ions allow the specific activation of calmodulin-stimulated enzymes. Biochemistry **20**, 3890-3897.

Haiech, J., Vallet, B., Robert, A., and Demaille, J.G. (1980). Ligand binding to macromolecules: determination of binding parameters by combined use of ligand buffers and flow dialysis; application to calcium-binding proteins. Anal. Biochem. **105**, 18-23.

Haley, A., Russell, A.J., Wood, N., Allan, A.C., Knight, M., Campbell, A.K., and Trewavas, A. J. (1995). Effects of mechanical signaling on plant cell cytosolic calcium. Proc. Natl. Acad. Sci. USA **92**, 4124-4128.

Hardie, R. (1996). Calcium signaling: setting store by calcium channels. Curr. Biol. 6, 1371-1373.

Harmon, A.C., Putnam-Evans, C., and Cormier, M.J. (1987). A calcium-dependent but calmodulin-independent protein kinase from soybean. Plant Physiol. **83**, 830-837.

Harmon, A.C., Yoo, B.-C., Lee, J.-Y., Zhang, Y., and Roberts, D.M. (1996). Molecular and biochemical properties of calmodulin-like domain protein kinases. In Protein phosphorylation in plants, Shewry, P.R., Halford, N.G., and Hooley, R. eds. Oxford University Press, pp. 267-277.

Harmon, A.C., Yoo, B.-C., and McCaffery, C. (1994). Pseudosubstrate inhibition of CDPK, a protein kinase with a calmodulin-like domain. Biochemistry **33**, 7278-7287.

Harper, J.F., Binder, B.M., and Sussman, M.R. (1993). Calcium and lipid regulation of an *Arabidopsis* protein kinase expressed in *Escherichia coli*. Biochemistry **32**, 3282-3290.

Harper, J.F., Huang, J.F., and Lloyd, S.J. (1994). Genetic Identification of an autoinhibitor in CDPK, a protein kinase with a calmodulin-like domain. Biochemistry **33**, 7267-7277.

Harper, J.F., Sussman, M.R., Schaller, G.E., Putnam-Evans, C., Charbonneau, H., and Harmon, A.C. (1991). A calcium-dependent protein kinase with a regulatory domain similar to calmodulin. Science **252**, 951-954.

Hashimoto, Y., Nakayama, T., Teramoto, T., Kato, H., Watanabe, T., Kinoshita, M., Tsukamoto, K., Tokunaga, K., Kurokawa, K., Nakanishi, S., Matsuda, Y., and Nonomura, Y. (1991). Potent and preferential inhibition of Ca^{2+}/calmodulin-dependent protein kinase II by K252a and its derivative, KT5926. Biochem. Biophys. Res. Commun. **181**, 423-429.

Heidorn, D.B., and Trewhella, J. (1988). Comparison of the crystal and solution structures of calmodulin and troponin C. Biochemistry **27**, 909-915.

Hepler, P.K., and Wayne, R.O. (1985). Calcium and plant development. Ann. Rev. Plant Physiol. **36**, 397-439.

Hidaka, H., Inagaki, M., Kawamoto, S., and Sasaki, Y. (1984). Inoquinolinesulfonamides, novel and potent inhibitors of cyclic nucleotide dependent protein kinase and protein kinase C. Biochemistry **23**, 5036-5041.

Hidaka, H., Watanabe, M., and Kobayashi, R. (1991). Properties and use of H-series compounds as protein kinase inhibitors. Meth. Enzymol. **201**, 328-339.

Hong, Y., Takano, M., Liu, C.-M., Gasch, A., Chye, M.-l., and Chua, N.-H. (1996). Expression of three members of the calcium-dependent protein kinase gene family in *Arabidopsis thaliana*. Plant Mol. Biol. **30**, 1259-1275.

Hrabak, E.M., Dickmann, L.J., Satterlee, J.S., and Sussman, M.R. (1996). Characterization of eight new members of the calmodulin-like domain protein kinase gene family from *Arabidopsis thaliana*. Plant Mol. Biol. **31**, 405-412.

Hsiao, K.-C. (1991). A fast and simple procedure for sequencing double stranded DNA with Sequenase. Nuc. Acids Res. **19**, 2787.

Ikura, M. (1996). Calcium binding and conformational response in EF-hand proteins. Trends Biochem. Sci. **21**, 14-17.

Ikura, M., Clore, G., Gronenborn, A., Zhu, G., Klee, C., and Bax, A. (1992). Solution structure of a calmodulin-target peptide complex by multidimensional NMR. Science **256**, 632-638.

James, P., Vorherr, T., and Carafoli, E. (1995). Calmodulin-binding domains: just two faced or multi-faceted? Trends Biochem. Sci. **20**, 38-42.

Johannes, E. and Sanders, D. (1995). Lumenal calcium modulates unitary conductance and gating of a plant vacuolar calcium release channel. J. Membrane Biol. **146**, 211-224.

Kakalis, L.T., Kennedy, M., Sikkink, R., Rusnak, F., and Armitage, I.M. (1995). Characterizations of calcium-binding sites of calcineurin B. FEBS Lett. **362**, 55-58.

Kase, H., Iwahash, K., Nakanishi, S., matsuda, Y., Yamada, K., Takahashi, M., Murakata, C., Sato, A., and Kaneko, M. (1987). K-252 Compounds, novel and potent inhibitors of protein kinase C and cyclic nucleotide-dependent protein kinases. Biochem. Biophys. Res. Commun. **142**, 436-440.

Kawasaki, T., Hayashida, N., Bada, T., Shinozaki, K., and Shimada, H. (1993). The gene encoding a calcium-dependent protein kinase located near the *sbe1* gene encoding starch branching enzyme I is specifically expressed in developing rice seeds. Gene **129**, 183-189.

Kearns, E.V. and Assmann, S.M. (1993). The guard cell environment connection. Plant Physiol. **102**, 711-715.

Kelley, W.B., Esser, J.E., and Schroeder, J.I. (1995). Effects of cytosolic calcium and limited, possible dual, effects of G protein modulators on guard cell inward potassium channels. Plant J. **8**, 479-489.

Klotz, I.M. (1983). Ligand-receptor interactions: what we can and cannot learn from binding measurements. Trends Pharmacol. Sci. 4, 253-255.

Knight, H., Trewavas, A., and Knight, M.R. (1996). Cold calcium signaling in *Arabidopsis* involves two cellular pools and a change in calcium signature after acclimation. Plant Cell **8**, 489-503.

Knight, M.R., Cambell, A.K., Smith, S.M., and Trewavas, A.J. (1991). Transgenic plant aequorin reports the effects of touch, cold-shock and elicitors on cytoplasmic calcium. Nature **352**, 524-526.

Knight, M.R., Heather, K., and Watkins, N.J. (1995). Calcium and generation of plant form. Phil. Trans. R. Soc. Lond . B. **350**, 83-86.

Knight, M.R., Smith, S.M., and Trewavas, A.J. (1992). Wind-induced plant motion immediately increases cytosolic calcium. Proc. Nat. Acad. Sci. USA **89**, 4967-4971.

Kretsinger, R.H. (1987). Calcium coordination and the calmodulin fold: divergent versus convergent evolution. Cold Spring Harbor Symp. on Quant. Biol. LII, 499-510.

Kretsinger, R.H. (1996). EF-hands reach out. Nature Struct. Biol. **3**, 12-53.

Lee, H.J., Tucker, E.B., Crain, R.C., Lee, Y. (1993). Stomatal opening is induced in epidermal peels of *Commelina communis* L. by GTP analogs or pertussis toxin. Plant Physiol. **102**, 95-100.

Levine, A., Pennell, R.I., Alvarez, M.E., Palmer, R., and Lamb, C. (1996). Calcium-mediated apoptosis in a plant hypersensitive disease resistance response. Curr. Biol. 6, 427-437.

Lindzen, E., and Choi, J.H. (1995). A carrot cDNA-encoding an atypical protein-kinase homologous to plant calcium-dependent protein-kinases. Plant Mol. Biol. 28, 785-797.

Linse, S., Helmersson, A., and Forsen, S. (1991). Calcium binding to calmodulin and its globular domains. J. Biol. Chem. 266, 8050-8054.

Luan, S., Li, W., Rusnak, F., Assmann, S.M., and Schreiber, S.L. (1993). Immunosuppressants implicate protein phosphatase regulation of K^+ channels in guard cells. Proc. Natl. Acad. Sci. USA 90, 2202-2206.

Malho, R., and Trewavas, A.J. (1996). Localized apical increases of cytosolic free calcium control pollen tube orientation. Plant Cell 8, 1935-1949.

Maune, J.F. (1992). Ca^{2+} binding and conformational changes in two series of point mutations to the individual Ca^{2+}-binding sites of calmodulin. J. Biol. Chem. 267, 5286-5295.

McAinsh, M.R., Brownlee, C., and Hetherington, A. (1992). Visualizing changes in cytosolic-free Ca^{2+} during the response of stomatal guard cells to abscisic acid. Plant Cell 4, 1113-1122.

McAinsh, M.R., Clayton, M., T.A., and Hetherington, A.M. (1996). Changes in stomatal behavior and guard cell cytosolic free calcium in response to oxidative stress. Plant Physiol. 111, 1031-1042.

McAinsh, M.R., Webb, A.A.R., Taylor, J.E., and Hetherington, A.M. (1995). Stimulus-induced oscillations in guard cell cytosolic free calcium. Plant Cell 7, 1207-1219.

McCarty, D. R. (1986). A simple method for extraction of RNA from maize tissues. Maize Genetics Coop. Letters 60, 61.

Meador, W.E., Means, A.R., and Quiocho, F.A. (1992). Target enzyme recognition by calmodulin: 2.4 A structure of a calmodulin-peptide complex. Science 257, 1251-1255.

Meador, W.E., Means, A.R., and Quiocho, F.A. (1993). Modulation of calmodulin plasticity in molecular recognition on the basis of X-ray structures. Science 262, 1718-1721.

Mikoshiba, K. (1993). Inositol 1, 4, 5-triphosphate receptor. Trends Pharmacol. Sci. **14**, 86-89.

Minta, A., Kao, J.P., and Tsiens, R.Y. (1989). Fluorescent indicators for cytosolic calcium based on rhodamine and fluorescein chromophores. J. Biol. Chem. **264**, 8171-8178.

Miyazaki, S. (1995). Inositol triphosphate receptor mediated spatiotemporal calcium signalling. Curr. Opin. Cell Biol. **7**, 190-196.

Mochly-Rosen, D. (1995). Localization of protein kinases by anchoring proteins: a theme in signal transduction. Science **268**, 247-250.

Moncrief, N.D., Kretzinger, R.H., Goodman, M. (1990). Evolution of EF-hand calcium-modulated proteins. I. Relationships based on amino acid sequences. J. Mol. Evol. **30**, 522-562.

Monroy, A.F., and Dhindsa, R.S. (1995). Low-temperature signal transduction: induction of cold acclimation-specific genes of alfalfa by calcium at 25 çC. Plant Cell **7**, 321-331.

Muir, S. R. and Sanders, D. (1996). Pharmacology of Ca^{2+} release from red beet microsomes suggests the presence of ryanodine receptor homologs in higher plants. FEBS Lett. **395**, 39-42.

Nakayama, S., and Kretsinger, R.H. (1994). Evolution of the EF-hand family of proteins. Annu. Rev. Biomol. Struct. **23**, 473-507.

Nauhaus, G., Bowler, C., Kern, R., and Chua, N.-H. (1993). Calcium/calmodulin-dependent and -independent phytochrome signal transduction pathways. Cell **73**, 937-952.

Neer, E.J. (1995). Heterotrimeric G proteins: organizers of transmembrane signals. Cell **80**, 249-257.

Olmsted, J.B. (1981). Affinity purification of antibodies from diazotized paper blots of heterogeneous protein samples. J. Biol. Chem. **256**, 11955-11957.

Olwin, B.B., and Storm, D. R. (1985). Calcium binding to complexes to calmodulin and calmodulin binding proteins. Biochemistry **24**, 8081-8086.

Parekh, A.B., Terlau, H., and Stuhmer, W. (1993). Depletion of $InsP_3$ stores activates a Ca^{2+} and K^+ current by means of a phosphatase and a diffusible messenger. Nature **364**, 814-818.

Patil, S., Takezawa, D., and Poovaiah, B.W. (1995). Chimeric plant calcium/calmodulin-dependent protein-kinase gene with a neural visinin-like calcium-binding domain. Proc. Natl. Acad. Sci. USA **92**, 4897-4901.

Persechini, A., and Kretsinger, R. (1988). The central helix of calmodulin functions as a flexible tether. J. Biol. Chem. **263**, 12175-12178.

Philosoph-Hadas, S., Meir, S., Rogenberger, I., and Halevy, A.H. (1996). Regulation of the gravitropic response and ethylene biosynthesis in gravistimulated snapdragon spikes by calcium chelators and ethylene inhibitors. Plant Physiol. **110**, 301-310.

Pierson, E.S., miller, D. D., Callaham, D.A., Shipley, A.M., Rivers, B.A., Cresti, M., and Hepler, P.K. (1994). Pollen tube growth is coupled to the extracellular calcium ion flux and the intracellular calcium gradient: effect of BAPTA-type buffers and hypertonic media. Plant Cell **6**, 1815-1838.

Pineros, M. and Tester, M. (1995). Characterization of a voltage-dependent Ca^{2+}-selective channel from wheat roots. Planta **195**, 478-488.

Pisa-Williamson, D., and Fuller, C.W. (1992). Glycerol tolerant DNA sequencing Gels. Comments, Promega **19**, 29-36.

Porumb, T. (1994). Determination of calcium-binding constants by flow dialysis. Anal. Biochem. **220**, 227-237.

Potter, J.D., Strang-Brown, P., Walker, P., and Iida, S. (1983). Ca^{2+} binding to calmodulin. Meth. Enzymol. **102**, 135-143.

Price, A.H., Taylor, A., Ripley, S.J., Griffiths, A., and Trewavas, A.J. (1994). Oxidative signals in tobacco increase cytosolic calcium. Plant Cell **6**, 1301-1310.

Putnam-Evans, C., Harmon, A.C., Palevitz, B.A., Fechheimer, M., and Cormier, M.J. (1989). Calcium-dependent protein kinase is localized with F-actin in plant cells. Cell Motil. Cytoskel. **12**, 12-22.

Putnam-Evans, C.L., Harmon, A.C., and Cormier, M.J. (1990). Purification and characterization of a novel calcium-dependent protein kinase from soybean. Biochemistry **29**, 2488-2495.

Putney, J. W. (1993). Excitement about calcium signaling in inexcitable cells. Science **262**, 676-678.

Quail, P.H. (1991). Phytochrome: a light-activated molecular switch that regulates plant gene expression. Annu. Rev. Genet. **25**, 389-409.

Randriamampita, C. and Tsien, R. (1993). Emptying of intracellular Ca^{2+} stores releases a novel small messenger that stimulates Ca^{2+} influx. Nature **364**, 809-814.

Raz, V., and Fluhr, R. (1992). Calcium requirement for ethylene-dependent responses. Plant Cell **4**, 1123-1130.

Rens-Domiano, S. and Hamm, H.E. (1995). Structural and functional relationship of heterotrimeric G-Proteins. FASEB J. **9**, 1059-1066.

Roberts, D.M., and Harmon, A.C. (1992). Calcium-modulated proteins: Targets of intracellular calcium signals in higher plants. Annu. Rev. Plant Physiol. **43**, 375-414.

Rudd, J.J., Franklin, F.C.H., Lord, J.M., and Franklin-Tong, V.E. (1996). Increased phosphorylation of a 26-kD pollen protein is induced by the self-incompatibility response in *Papaver rhoeas*. Plant Cell **8**, 713-724.

Saitoh, M., Ishikawa, T., Matsushima, S., Naka, M., and Hidaka, H. (1987). Selective inhibition of catalytic activity of smooth muscle myosin light chain kinase. J. Biol. Chem. **262**, 7796-7801.

Sanders, D., Muir, S.R., and Allen, G.J. (1995). Ligand- and voltage-gated calcium release channels at the vacuolar membrane. Biochem. Soc. Trans. **23**, 856-861.

Sanger, F., Nicklen, S., and Coulson, A.R. (1977). DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. USA **74**, 5463-5467.

Schaller, G.E., Harmon, A.C., and Michael, R.S. (1992). Characterization of a calcium- and lipid-dependent protein kinase associated with the plasma membrane of oat. Biochemistry **31**, 1721-1727.

Schroeder, J.I., and Hagiwara, S. (1989). Cytosolic calcium regulates ion channels in the plasma membrane of *Vicia faba* guard cell. Nature **338**, 427-430.

Schroeder, J.I., and Hagiwara, S. (1990). Repetitive increases in cytosolic Ca^{2+} of guard cells by abscisic acid activation of non selective Ca^{2+} permeable channels. Proc. Nat. Acad. Sci. USA **87**, 9305-9309.

Schulman, H. (1993). The multifunctional Ca^{2+}/calmodulin-dependent protein kinases. Curr. Opin. Cell Biol. **5,** 247-253.

Sedbrook, J.C., Kronebusch, P.J., Borisy, G.G., Trewavas, A.J., and Masson, P. (1996). Transgenic aequorine reveals organ-specific cytosolic Ca^{2+} responses to anoxia in *Arabidopsis thaliana* seedlings. Plant Physiol. **111,** 243-257.

Shacklock, P. S., and Trewavas, A. J. (1992). Cytosolic free calcium mediates red light-induced photomorphogenesis. Nature **358,** 753-755.

Sitsapesan, R., McGarry, S. J., and Williams, A. J. (1995). Cyclic ADP-ribose, the ryanodine receptor and Ca^{2+} release. Trends Pharmacol. Sci. **16,** 386-391.

Smith, D.A. and Fisher, P.A. (1984). Identification, developmental regulation, and response to heat shock of two antigenically related forms of a major nuclear envelope protein in *Drosophila* Embryos: Application of an improved method for affinity purification of antibodies using polypeptides immobilized on nitrocellulose blots. J. Cell Biol. **99,** 20-28.

Son, M., Gunderson, R.E., and Nelson, D.L. (1993). A second member of the novel Ca^{2+}-dependent protein kinase family from *Paramecium tetraurelia*. J. Biol. Chem. **8,** 5940-5948.

Starovasnik, M., Davis, T.N., and Klevit, R.E. (1993). Similarities and differences between yeast and vertebrate calmodulin: an examination of the calcium-binding and structural properties of calmodulin from the yeast *Saccharomyces cerevisiae*. Biochemistry **32,** 3261-3270.

Stemmer, P., and Klee, C.B. (1994). Dual calcium ion regulation of calcineurin by calmodulin and calcineurin B. Biochemistry. 33, 6859-6866.

Studier, F.W., Rogenberg, A.H., and Dunn, J.J. (1990). Use of T7 RNA polymerase to direct the expression of cloned genes. Meth. Enzymol. **185,** 60-89.

Subbaiah, C.C., Zhang, J., and Sachs, M.M. (1994). Involvement of intracellular calcium in anaerobic gene expression and survival of maize seedlings. Plant Physiol. **105,** 369-376.

Suen, K.-L., and Choi, J.H. (1991). Isolation and sequence analysis of a cDNA clone for a carrot calcium-dependent protein kinase: homology to

calcium/calmodulin-dependent protein kinase and to calmodulin. Plant Mol. Biol. **17**, 581-590.

Sumi, M., Kiuch, K., Ishikawa, T., Ishii, A., Hagiwara, M., Nagatsu, T., and Hidaka, H. (1991). The newly synthesized selective Ca^{2+}/calmodulin dependent protein kinase II inhibitor KN-93 reduces dopamine contents in PC12h cells. Biochem. Biophys. Res. Commun. **181**, 968-975.

Takezawa, D., Patil, S., Bhatia, A., and Poovaiah, B.W. (1996a). Calcium-dependent protein kinase genes in corn roots. J. Plant Physiol. **149**, 329-335.

Takezawa, D., Ramachandiran, S., Paranjape, V., and Poovaiah, B.W. (1996b). Dual regulation of a chimeric plant serine threonine kinase by calcium and calcium-calmodulin. J. Biol. Chem. **271**, 8126-8132.

Tamaoki, T. (1991). Use and specificity of staurosporine, UCN-01, and calphostin C as protein kinase inhibitors. Meth. Enzymol. **201**, 340-347.

Taylor, A.R., Manison, F.H., Fernandez, C., Wood, J., Brownlee, C. (1996). Spatial organization of calcium signaling involved in cell volume control in the *Fucus rhizoid*. Plant Cell **8**, 2015-2031.

Thomas, A. P., Bird, G. ST. J., Hajnoczky, G., Robb-Gaspers, L.D., and Putney, J. W. (1996). Spatial and temporal aspects of cellular calcium signaling. FASEB J. **10**, 1505-1517.

Thuleau, P., Ward, J.M., Ranjeva, R., and Schroeder, J.I. (1994). Voltage-dependent calcium-permeable channels in the plasma membrane of a higher plant cell. EMBO J. **13**, 2970-2975.

Torok, K., and Whitaker, M. (1994). Taking a long, hard look at calmodulin's warm embrace. Bio Essays **16**, 221-224.

Trewavas, A., and Knight, M. (1994). Mechanical signaling, calcium and plant form. Plant Mol. Biol. **26**, 1329-1341.

Trewavas, A., Read, N., Campbell, A.K., and Knight, M. (1996). Transduction of Ca^{2+} signals in plant cells and compartmentalization of the Ca^{2+} signal. Biochem. Soc. Trans. **24**, 971-974.

Urao, T., Katagiri, T., Mizoguchi, T., Yamaguchi-Shinozaki, K., Hayashida, N., and Shinozaki, K. (1994). Two genes that encode Ca^{2+}-dependent

protein kinases are induced by drought and high-salt stresses in *Arabidopsis thaliana*. Mol. Gen. Genet. **244**, 331-340.

Verhey, S.D., Gaiser, J.C., and Lomax, T.L. (1993). Protein kinases in zucchini. Characterization of calcium-requiring plasma membrane kinases. Plant Physiol. **103**, 413-419.

Waltersson, Y., Linse, S., Brodin, S., and Grundstrom, T. (1993). Mutational effects on the cooperativity of Ca^{2+} binding in calmodulin. Biochemistry 32, 7866-7871.

Ward, J.M., and Schroeder, J.I. (1994). Calcium-activated K^+ channels and calcium-induced calcium release by slow vacuolar ion channels in guard cell vacuoles implicated in the control of stomatal closure. Plant Cell **6**, 669-683.

Webb, A.A.R., MaAnish, M.R., Mansfield, T.A., and Hetherington, A.M. (1996). Carbon dioxide induces increases in guard cell cytosolic free calcium. Plant J. **9**, 297-304.

Womack, F.C., and Colowick, F.C. (1973). Rapid measurement of binding of ligands by rate of dialysis. Meth. Enzymol. **25**, 464-471.

Yazawa, M., Ikura, M., Hikichi, K., Ying, L., and Yagi, K. (1987). Communication between two globular domains of calmodulin in the presence of mastoparan or caldesmon fragment. Ca^{2+} binding and 1H NMR. J. Biol. Chem. **262**, 10951-10954.

Yazawa, M., Vorherr, T., James, P., Carafoli, E., and Yagi, K. (1992). Binding of calcium by calmodulin: Influence of the calmodulin binding domain of the plasma membrane calcium pump. Biochemistry **31**, 3171-3176.

Yoo, B.-C., and Harmon, A.C. (1996). Intramolecular binding contributes to the activation of CDPK, a protein kinase with a calmodulin-like domain. Biochemistry 35, 12029-12037

Zhao, Y., Kappes, B., and Franklin, R.M. (1993). Gene structure and expression of an unusual protein kinase from *Plasmodium falciparum* homologous at its carboxyl terminus with the EF hand calcium-binding proteins. J. Biol. Chem. **268**, 4347-4354.

Zhao, Y., Pokutta, S., Maurer, P., Lindt, M., Franklin, R.M., and Kappes, B. (1994). Calcium-binding properties of a calcium-dependent protein kinase from *Plasmodium falciparum* and the significance of individual calcium-binding sites for kinase activation. Biochemistry 33, 3714-3721.

Zocchi, G., and De Nish, P. (1996). Physiological and biochemical mechanisms involved in the response to abscisic acid in maize coleoptile. Plant Cell Physiol. **37**, 840-846.

BIOGRAPHICAL SKETCH

Jung-Youn Lee was born May 11, 1966, in an island on West Sea of Korea. Her family soon moved to Seoul, Korea after her birth. She attended Korea University in Seoul, Korea, receiving a Bachelor of Agriculture in February of 1990 from Department of Horticulture and pursued the graduate study at the University of Florida in the fall of 1990 together with her husband, Byung-Chun. She earned the first Master's degree from interdisciplinary program of Plant Molecular and Cellular Biology in August, 1992 under the supervision of Dr. Curt L. Hannah. She then moved to Dr. Alice Harmon's lab to continue the graduate study for the Ph. D. degree. While working as a Ph. D. student she gave birth to their adorable son, Jaewoong, who is now three years old. She will continue her career as a Scientist.

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

Alice C. Harmon, Chair
Associate Professor of Plant
Molecular and Cellular Biology

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

Robert J. Ferl
Professor of Plant Molecular and
Cellular Biology

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

William B. Gurley
Associate Professor of Plant
Molecular and Cellular Biology

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

David A. Jones
Professor of Botany

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

Donald R. McCarty
Associate Professor of Plant
Molecular and Cellular Biology

This dissertation was submitted to the Graduate Faculty of the Program in Plant Molecular and Cellular Biology in the College of Liberal Arts and Sciences and to the Graduate School and was accepted as partial fulfillment of the requirements for the degree of Doctor of Philosophy.

May, 1997

Dean, Graduate School